SECONDARY PRODUCT BIOSYNTHESIS IN TISSUE CULTURES OF ONION (*ALLIUM CEPA* L.)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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'Indeed, of the many gifts that Europe owes to Asia, none, I imagine, have done more to reconcile man to a life of penury, than the vine and onion family. The labourer in Greek fields, with the traditional crust of bread, a skin of wine and a clove of garlic, could support his hard labour on those sunswept slopes. Even today his brother in England finds his lunch of bread and cheese a little tasteless without an onion'.

Bunyard.

CONTENTS

List of abbreviations

Ala	alanine
All Cys	S - allyl cysteine
All Cy SO	S - allyl cystcine sulphoxide
All SH	allyl mercaptan
Arg	arginine
Asp	aspartic acid
Asn	asparagine
5-BU	5-bromouracil
BUdR	5-bromodeoxyuridine
CPC	S-(2-carboxypropyl) cysteine
CPG	S-(2-carboxypropyl) glutathione
Cys	cysteine
EMS	ethyl methane sulphonate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GSH	glutathione
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
MCW	methanol/chloroform/water
Me Cys	S-methyl cysteine
Me Cy SO	S-methyl cysteine sulphoxide
Me SH	methyl mercaptan
Met	methionine

MMS	S-methylmethionine sulphonium ion
NAA	naphthalene - acetic acid
NTG	N-methyl-N'-nitro-N-nitroso guanidine
Pr Cys	S-propyl cysteine
Pr Cy SO	S-propyl cysteine sulphoxide
Pren Cys	S-(propen-1-yl) cysteine
Pren Cy SO	S-(propen-1-yl) cysteine sulphoxide
Pro	proline
Pr SH	propyl mercaptan
Ser	serine
Thr	threonine
TLC	thin layer chromatography
Trp	tryptophan
Tyr	tyrosine
Val	valine
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(3-methyl-2-buten-1-yl amino purine)

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Summary

Callus cultures derived from seedling roots of onion were used for the study of secondary product formation in cultured tissues.

Preliminary experiments indicated that the pathway leading to Pren Cy SO formation was blocked in callus but the results of later work in which radioactively labelled intermediates were fed to callus showed that the pathway was operating. They also indicated that the Pren Cy SO so formed may be bound with glutamic acid to form peptides which are then unavailable for flavour production when the tissue is crushed.

Amino acid analysis of extracts of callus material which had been exogenously supplied with the intermediates CPC and Pren Cys indicated that the callus was able to take up these compounds and carry out their conversion to Pren Cy SO. Furthermore, it was demonstrated that the callus responded to the presence of Pren Cy SO, which arose by uptake of exogenously supplied Pren Cy SO or by conversion of CPC and Pren Cys, by synthesising more Pren Cy SO.

Transfer of callus to the appropriate differentiating media resulted in the induction of true roots and shoots with the accompanying development of flavour production. Callus-produced roots and rooting callus, when crushed, produced a distinct lachrymatory effect although only a faint onion smell was detected in crushed callus shoot material. The presence of Pren Cy SO in callus produced roots and shoots was demonstrated by TLC - electrophoresis.

Preliminary experiments showed that flavour precursors were present at all stages of seedling growth, including the dormant seed.

Comparison of onion bulb and callus cells by electron microscopy showed the presence of specialised vesicles which were characteristic of onion tissue but were absent from callus cells. These vesicles are the suggested sites of synthesis and/or accumulation of the secondary products of onion.

Subcellular fractionation of onion bulb and callus tissue homogenates showed that the alliinase enzyme was always associated with the soluble fraction. The lysosomes or the central vacuole are possible sites of accumulation for alliinase, although this enzyme may exist freely in the cytoplasm. The intracellular sites of synthesis and accumulation of the flavour precursors were not located.

Attempts to produce mutants from callus cultures which were able to synthesise levels of secondary products approaching those of the intact plant were unsuccessful. Successful production of mutant cell lines and selection of high yielding clones requires the development of a suspension culture.

The industrial potential of these findings is discussed.

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CHAPTER 1

GENERAL INTRODUCTION.

The onion (*Allium cepa* L.) is one of the most important and widely used vegetables in the world, not only as a spice but also as a popular herbal remedy. Records of the use of plants as a source of medicinal drugs dates back as far as 4000BC (Nickell 1959) and, like many other strong-smelling plants, the onion has had a traditional place in folk medicine for centuries (Stoll and Seebeck 1951). It was interest in the use of onion and other members of the *Allium* family in the treatment of various ailments and diseases that prompted early workers to search for the active principle responsible for the antimicrobial activity of these plants. Compounds active against a wide variety of micro-organisms (Abdou et al. 1972) have been found in the dry scales of the mature onion bulb (Link and Walker 1933) and in extracts of onion flesh (Hatfield et al. 1948). The volatile compounds which are produced by enzymic degradation of inactive precursors when onion tissue is damaged were also shown to play an important role in disease resistance (Virtanen and Matikkala 1959). Similar investigations have been carried out in recent years by Bordia et al. (1977) who found that the essential oils of onion were effective in reducing the rise in serum cholesterol and serum triglyceride levels which resulted from feeding high levels of cholesterol to rabbits. It was concluded that since the essential oils of onion may protect against experimental atherosclerosis, they may have a potential role in the treatment of this condition.

Interest in the culinary use of onion was the second major reason for research into the chemical composition of this plant. The onion is becoming increasingly important in the manufacture of 'convenience foods' such as dehydrated food products in the form of dried onion and flavour preparations such as onion salt. As a result, attempts have been made to understand the chemistry of the flavour compounds and the changes which occur as the vegetables are processed. Onions are used mainly as flavouring since the nutritional value in terms of protein, fat and carbohydrate content of the raw vegetable is very low (McCance and Widdowson 1967). However, onions are a rich source of some minerals and vitamins such as calcium, iron, vitamin A, thiamine, riboflavin and ascorbic acid (Abraham et al. 1976).

To supply the needs of the food industry a large proportion of the onions have to be imported. In recent years in the U.K. only 20% of the onion requirement was met by the British harvest, whilst the remaining 80% was imported from countries such as Spain, Portugal, Egypt, Canada (Min. Ag. Fish Food 1969) and the Netherlands (Min. Ag. Fish Food, 1967). The U.K. is the largest import market in the world, importing approximately 220 thousand tonnes onions per year (Jones and Mann 1963). This is mainly due to the unsuitable, post-harvest climatic conditions which prevail in the U.K. and hence the high level of wastage during bulb storage. Due to the costs involved in importation of vegetables, e.g. import duties, time involved and transport costs, it would be ..

advantageous if onion flavour compounds could be produced 'in vitro' by cultured tissues to supply at least part of the commercial food processors' requirements. This approach would be more acceptable than the use of synthetic flavour compounds since much legislation governs the use of such food additives (Schutte 1974) and more economical than extraction of flavour compounds from imported bulbs. For these reasons, the techniques of tissue culture applied to the production of secondary products such as the flavour compounds in onion, does have some potential.

The potential of plant cell culture for the biosynthesis of commercially important plant constituents was first realised in the early 1950s (Puhan and Martin 1971) and large scale production from cellsuspensions is now possible using similar batch culture techniques to those currently in use with micro-organisms. These processes have become fully automated with continual replenishment of nutrient medium so minimal time and attention is required (Mandels 1972).

One of the advantages of large scale tissue culture in the synthesis of secondary plant products is the fact that the system can be controlled precisely and all conditions of growth such as light, temperature, pH and availability of individual nutrients can be varied as required. The system also remains independent of factors which affect the supply of raw materials (Klein 1960) such as damage in shipment or storage, dependence on the weather, quality and uniformity of raw materials and the need for an ever increasing supply.

One of the main drawbacks of the method is that in many cases the yield of the desired product is much lower than that of the intact plant or the product may be absent altogether. Cultured cells sometimes produce a different pattern of secondary compounds to those from which the tissue was derived, which may in themselves be economically important products since they are often modifications of those found in the higher plant.

Steward et al. (1964) stated that "....each cell of the plant....has the genetic complement which is necessary to produce the whole organism.....". Thus, it is likely, in theory, that any product of the whole plant can be produced by cells derived from that plant, so it should be possible to control the production and yield of specific compounds in cultures by varying the growth conditions and supply of appropriate biosynthetic precursors. Many attempts have been made to induce secondary product synthesis in cultured cells by alteration of the medium composition (Amorim et al. 1977), growth conditions such as light (Kadkade and Seibert 1977), presence of growth factors such as auxins (Gamborg et al. 1970, Tabata et al. 1975), gibberellins (Gibson and French, 1964) and cytokinins (Skoog and Montaldi 1961), induction of morphogenesis (Tabata et al. 1972, Hiraoka and Tabata 1974) and supply of appropriate biosynthetic precursors (Kaul et al. 1969, Sairam and Khanna 1971, Tabata et al. 1971).

The term 'secondary products' as used here was first used to describe compounds which are not apparently essential to the

normal metabolism of the plants which produce them (Hegnauer 1975) and so they are not primary metabolites. Secondary compounds have been found in some fungi, bacteria and marine animals but the majority of them are of higher plant origin (Bell 1976, Swain 1977). They are not distributed uniformly throughout the plant kingdom and the occurrence of particular secondary compounds in related species was found to be a useful taxonomic character (Kjaer 1966, Gibbs 1974). For example, it was reported by Saghir et al. (1965) that whilst the total sulphide concentration may vary, the proportions of alkyl sulphide radicals in the vapours of various common Alliums were not affected by the habitat, stage of growth or plant part and that these ratios remained the same within a particular species. Further work by Bernhard in 1970 showed that the patterns of sulphur compounds were similar for members of a particular taxonomic group of Alliums, whereas the patterns differed significantly with different plant groups.

Some secondary products may be present in large amounts in the plant e.g. rubber in guayale (Parthenium argentatum) may account for 20% of the dry weight of the plant (Street and Cockburn 1972) and whilst some are present throughout an individual plant, others may be synthesised or accumulated in particular cells or tissues only at certain times during the life of the plant.

The function of secondary products is as yet unknown and is the subject of many review articles (Fraenkel 1969, Fowden 1974, Bell 1976, Seigler and Price 1976, Swain 1977). It seems unlikely that a plant would divert a large part of its reserves, biosynthetic capacity and storage space for the accumulation of compounds which are not necessary for primary metabolism. It was suggested by Bell (1976) that secondary compounds first arose as waste products which remained as chemical constituents of certain genera because they conferred a selective advantage upon the plant, perhaps by rendering it toxic to predators or parasites. The functions usually ascribed to secondary compounds include those of waste products, storage materials (Luckner 1974) or protective agents (Swain 1977).

Secondary compounds are usually grouped according to chemical structure and include alkaloids, amino acids, cyanogenic glycosides, flavonoids, phenolic acids, terpenes and steroids (Swain 1977). The group of interest in this investigation is that of the non-protein or 'uncommon' amino acids, so named because they always exist as free moieties or as simple condensation products such as γ-glutamyl derivatives (Thompson et al. 1962). They are never found as constituents of protein in the plants which produce them but if introduced into a species to which it is foreign, incorporation of an uncommon amino acid into protein may occur (Bell 1976). Over two hundred non-protein amino acids have been isolated and characterised in plant tissues, an overwhelming number compared

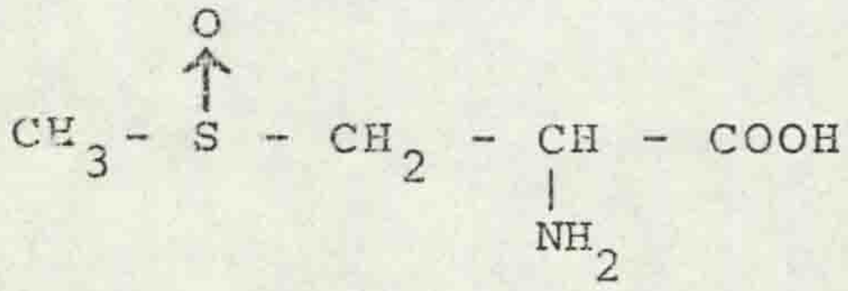
with only fifteen or sixteen of the twenty protein amino acids normally found in plant extracts. A large number of the non-protein amino acids are sulphur containing derivatives of cysteine (Fowden 1974) and it is to this sub-group that the flavour compounds of the Allium family belong. The degradation of these S-substituted cysteines is responsible for the characteristic onion smell which results when the tissue is damaged (Harborne 1973). Similarly in the Cruciferae, the flavours of mustard and radish are due to the presence of sulphur containing glucosinolates (mustard oil glycosides).

Non-protein amino acids may be synthesised in one of three ways (Bell 1976). They may be formed by a) modification of existing protein amino acids, b) modification of the bio-synthetic pathways normally associated with primary metabolism or c) synthesised by completely different routes. The flavour compounds of the onion are synthesised by modification of the protein amino acid, cysteine, by an extension of the existing primary metabolic pathway.

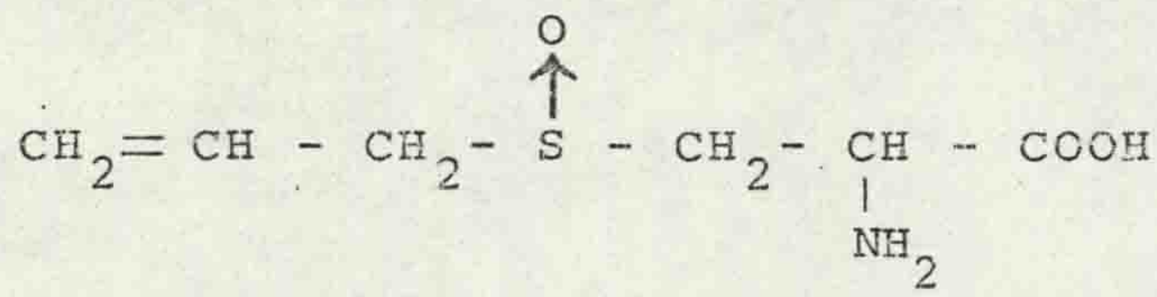
Investigation of onion flavour first started in the late nineteenth century when Semmler (1892) analysed steam distilled onion oils. Continued interest in the search for the antimicrobial principle of garlic led to the isolation of alliin by Cavallito and his co-workers (Cavallito and Bailey 1944, Cavallito et al. 1944). Following this, Stoll

and Seebeck (1951) carried out an extensive investigation into the characterisation and properties of alliin which they identified as allyl cysteine sulphoxide. The elucidation of the remaining flavour compounds of onion and garlic tissues was mainly due to the analytical work of Virtanen and co-workers and the synthesis work of Carson and his colleagues during the period from 1955 to 1970. Much of this work has been reviewed in various articles on vegetable flavours (MacLeod 1970, Abraham et al. 1976, Whitaker 1976). They describe the four main compounds in the Allium family which are responsible for the flavour and odour of these vegetables (Virtanen 1965), namely, S-allyl cysteine sulphoxide (All Cy SO) (Matsukawa et al. 1953, Saghir et al. 1964, Bernhard 1969), S-methyl cysteine sulphoxide (Me Cy SO) (Virtanen and Matikkala 1959, Carson and Wong 1961) S-propyl cysteine sulphoxide (Pr Cy SO) (Kuon and Bernhard 1963, Ettala and Virtanen 1962) and S-propenyl cysteine sulphoxide (Pren Cy SO) (Spare and Virtanen 1963, Virtanen and Spare 1961, 1962). The structure of these four compounds is shown in Fig. 1.1. The amounts and proportions of each of the compounds varies with the plant species, thus providing the distinctive flavours and odours of closely related species. All Cy SO is the predominant compound in garlic (Allium sativum) whilst Me Cy SO produces the characteristic cabbage like odour of many of the Cruciferae. Me Cy SO is the most widely distributed compound of the four, being found in varying amounts in most of the Alliums as well as some members of the Liliaceae, Compositae

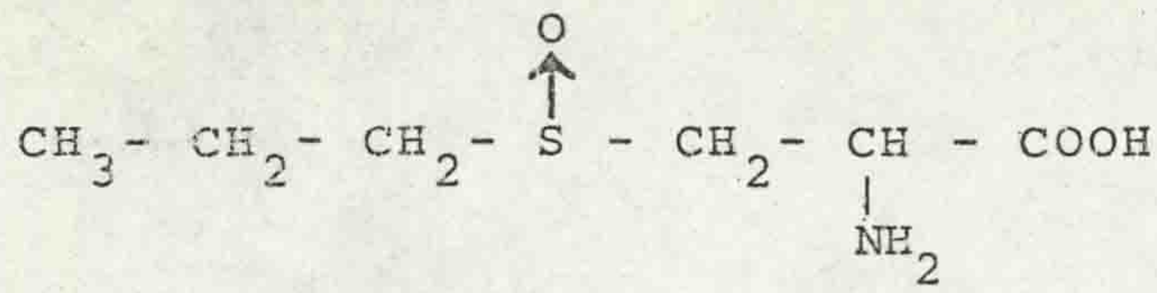
Fig. 1.1. Chemical structure of the four main flavour precursor compounds found in onion tissues.



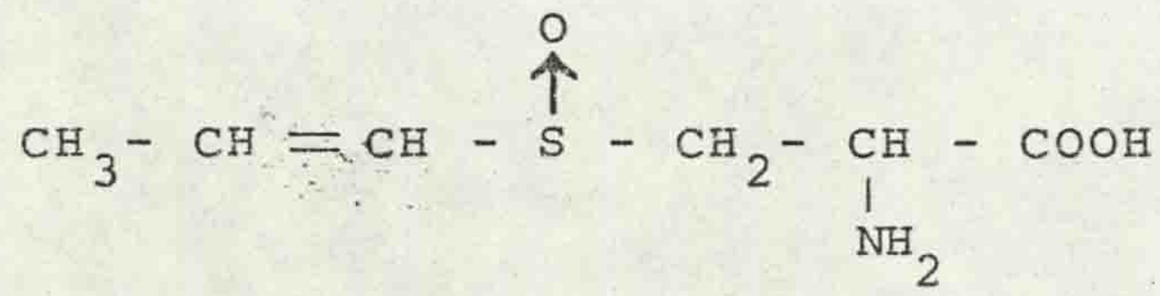
(+)-S-Methyl-L-Cysteine Sulphoxide, (MeCySO).



(+)-S-Allyl-L-Cysteine Sulphoxide, (Alliin, AllCySO).



(+)-S-Propyl-L-Cysteine Sulphoxide, (PrCySO).



Trans-(+)-S-(propen-1-yl)-L-Cysteine Sulphoxide,

(Trans-PrenCySO).

and Leguminosae (Whitaker 1976). Pr Cy SO is only found at low levels and has a more restricted distribution, being confined to the Alliums and one or two closely related species. Pren Cy So is widely distributed throughout the Allium family and is present in large quantities in the onion. The other three compounds are also present in onion tissue but Pren Cy So predominates.

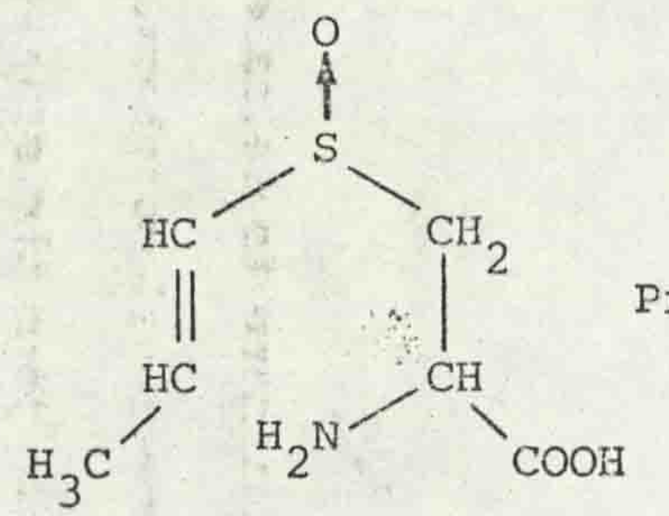
Onion flavour is now a classic example of enzymic flavour production in which the initial products of the reaction are highly unstable and undergo further changes (Carson 1967). The final products of the reaction are volatile, sulphur containing compounds which are ultimately responsible for the organoleptic characteristics of each species. The substrates of the enzyme alliinase, the flavour precursor compounds, are the four alkyl and alkenyl cysteine sulphoxides described above. Reaction between enzyme and substrate only occurs when onion tissue is damaged or cut, so the two reactants must be separated in some way, probably by compartmentalisation of one or both reactants.

Enzymic degradation of Pren Cy So, the principle flavour precursor in onion, is shown in Fig. 1.2. The initial product of the reaction is propenyl sulphenic acid which is highly unstable with a half life of ninety seconds (Moisio et al. 1962). This compound undergoes spontaneous rearrangement to form thiopropanal sulphoxide which is the lachrymatory factor of onion tissue. Degradation of the

Fig. 1.2 Reactions of Pren Cy SO:

a) Degradation by the enzyme alliinase to release the lachrymatory factor thiopropanal sulphoxide

b) Rearrangement of Pren Cy SO to form the stable compound, cycloalliin.



Propenyl Cysteine Sulphoxide

Alliinase

H_2O

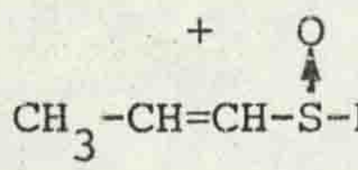
a

NH_3

+

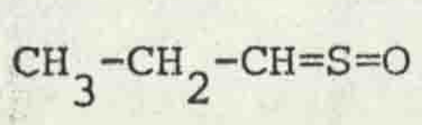
$CH_3.CO.COOH$ Pyruvate

+



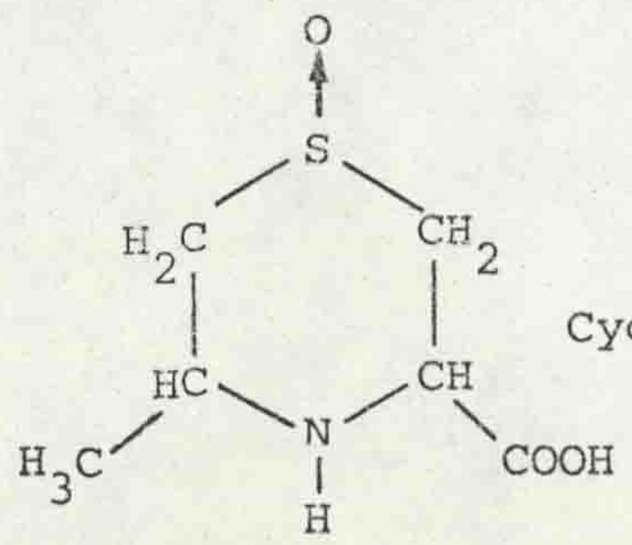
Propenyl sulphenic acid

Spontaneous rearrangement



Thiopropanal sulphoxide

b



Cycloalliin

remaining three flavour precursors differs in that the initial product formed is a thiosulphinate (Freeman and Whenham 1976) which readily disproportionates at room temperature to form thiosulphonates and disulphides. In each case, pyruvate is a stable end product of the reaction. Determination of enzymically produced pyruvate therefore provides a simple and rapid assay for the estimation of flavour precursor content and hence flavour intensity. Many investigations have been carried out concerning the nature of the volatile compounds produced by crushed tissues of onion and garlic and much of this work has been reported in recent review articles (Bernhard 1969, Schwimmer and Friedman 1972, Shankaranarayana et al. 1974).

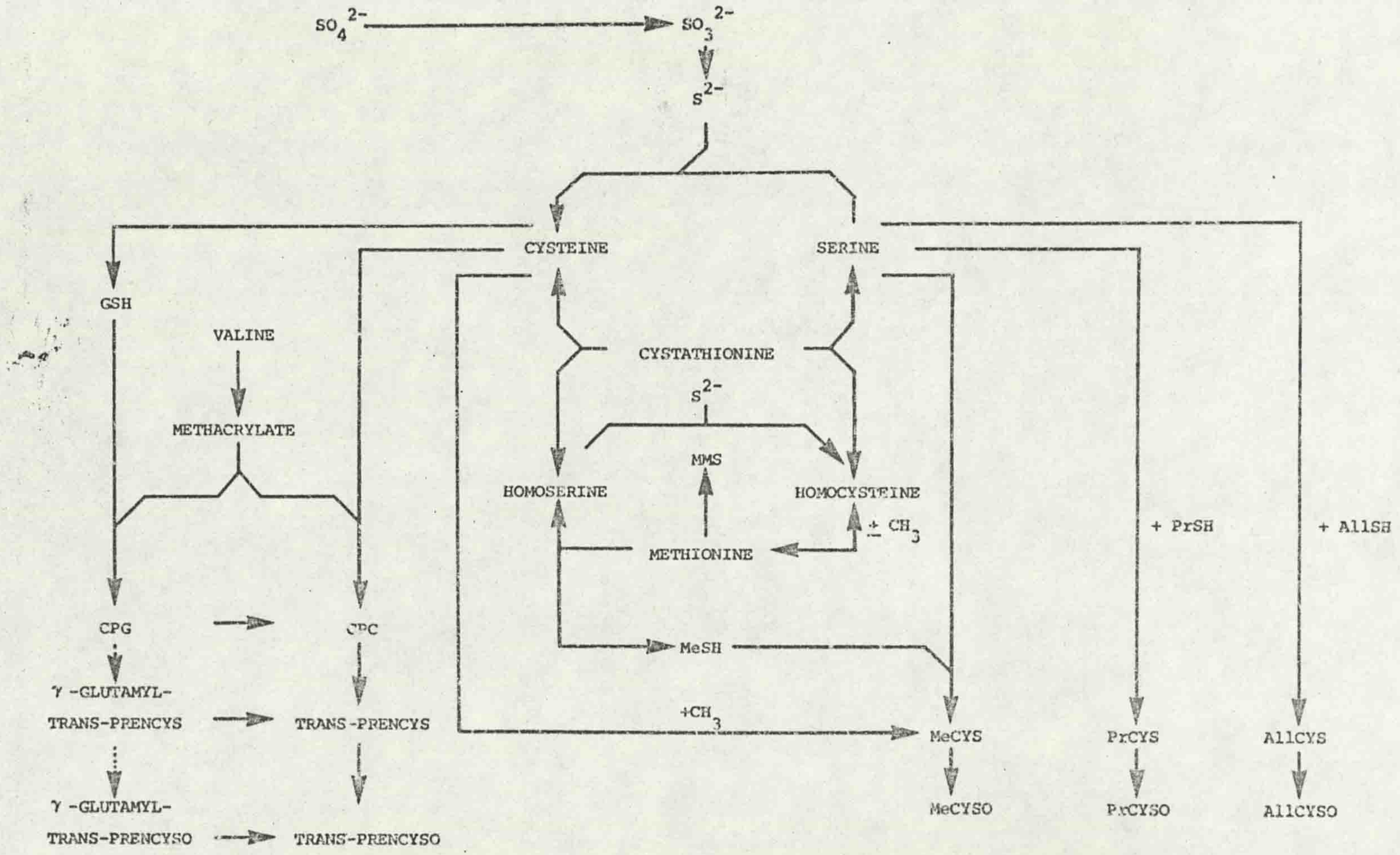
Another interesting compound isolated from onion tissues by Virtanen and Matikkala (1958, 1959) was the stable cyclic amino acid, cycloalliin (Fig. 1.2). This compound is present in intact onion tissue but it is also produced from Pren Cy So as an artifact during isolation. However, cycloalliin is inert to the enzyme alliinase and so it makes no contribution to onion flavour, a fact for which there is no satisfactory explanation. Approximately 50% of the Pren Cy So in onion tissue is in the form of a γ-glutamyl peptide and so would appear to be unavailable for flavour production. γ-glutamyl transpeptidase enzymes are present in onion leaves and sprouting bulbs (Matikkala and Virtanen 1965, Austin and Schwimmer 1971) but not in dormant bulbs. These enzymes presumably release the Pren Cy SO and render it available for enzymic degradation. However the role of these γ-glutamyl peptides in the onion

bulb and the nature of their synthesis and control 'in vivo' has not yet been determined.

The biosynthesis of the flavour precursor compounds, with special reference to Prcn Cy SO, was finally elucidated by Granroth in 1970. By feeding radioactively labelled sulphate to onion bulb slices and shoot tips, he was able to show that Pren Cy SO was produced by a unique pathway from valine and cysteine whilst the three alkyl cysteine sulphoxides were synthesised by identical routes from serine. A general scheme for the metabolism of the four flavour precursors is shown in Fig. 1.3.

The work reported in this thesis is a continuation of that by Selby and Collin (1976) in which they attempted to select callus clones which had the capacity for production of high levels of flavour compounds i.e. high yielding clones. They used three varieties of onion with different flavour strengths but found that all callus clones contained approximately the same low level of flavour production, with a maximum of 10% of the level found in the intact plant. Similar findings, based on an extensive series of chemical, gas-chromatographic and sensory tests were reported by Davey et al. (1974) and Freeman et al. (1974) where they concluded that onion callus tissue failed to produce significant amounts of the characteristic flavour compounds. Since onion flavour compounds are present in all parts of the plant (Saghir et al. 1965, Becker and Schuphan 1975) and not confined to any particular areas of synthesis or

Fig. 1.3 Metabolic relationships of some of the sulphur compounds found in the onion, as proposed by Granroth (1970).


SO_4^{2-}
SO_3^{2-}
S^{2-}
CYSTEINE
SERINE
GSH
VALINE
METHACRYLATE
CYSTATHIONINE
S^{2-}
MMS
HOMOSERINE
HOMOCYSTEINE
$\pm CH_3$
METHIONINE
CPG
CPC
MeSH
γ -GLUTAMYL-
TRANS-PRENCYS
TRANS-PRENCYS
$+CH_3$
MeCYS
+ PrSH
+ AllSH
γ -GLUTAMYL-
TRANS-PRENCYSO
TRANS-PRENCYSO
MeCYSO
PrCYS
AllCYS
PrCYSO
AllCYSO

accumulation, it was considered most likely that cultured cells of onion would have the capacity to synthesise secondary products. Unfortunately, this was not true of cultured onion tissue. However, Freeman et al. (1974) found that differentiation of roots from callus tissue resulted in the return of some aspects of flavour production although the lachrymatory effect was still absent. These findings were in agreement with the results of cultured tissues in general where it is often found that whilst secondary products are present at low levels or absent from callus tissue, initiation of morphogenesis in some way stimulates secondary compound synthesis (Krikorian and Steward 1969). Onion callus cultures have been induced to form shoots (Dunstan 1977) but no chemical analyses were done on the redifferentiated tissues.

It was suggested by Selby and Collin (Personal communication) that the low level of flavour compounds present in onion callus tissue was due to the three less important flavour compounds, while it was the main one, Pren Cy SO, that was absent. Preliminary experiments (Selby and Collin, Unpublished data) also indicated that the biosynthetic pathway of Pren Cy SO formation in callus tissue was blocked at one particular step which was the synthesis of CPC. The tissue culture of onion was therefore an ideal system upon which to work, since the metabolic pathways of flavour compound

synthesis were well established and documented and the lack or reduction of flavour synthesis in callus was thought to be due to failure of operation of a particular step in the biosynthetic pathway. Should it be possible to establish more definitely the reasons for reduced secondary product synthesis in onion callus cultures, the techniques used and results obtained might be of value in solving similar problems in other cultured tissue systems where secondary product synthesis is low.

Research Programme

Four main lines of approach to the problem were selected.

1. To determine the reason for reduced flavour production in callus tissue the biosynthetic pathways in both callus and actively growing onion tissue were compared by using precursor feeding techniques and radio-active labelling of intermediates.The metabolism of the precursors was examined by TLC-electrophoresis, auto-radiography and amino acid analysis.

2. To show the effect of morphogenesis on flavour production in callus cultures, onion callus was induced to form roots and shoots and the flavour compound contents of roots, shoots, differentiated and undifferentiated callus tissues were examined by TLC-electrophoresis, amino acid analyses and measurement of enzymically produced pyruvate. For comparison with normal differentiating systems, the roots and shoots of onion seedlings and a sprouting bulb were also analysed.

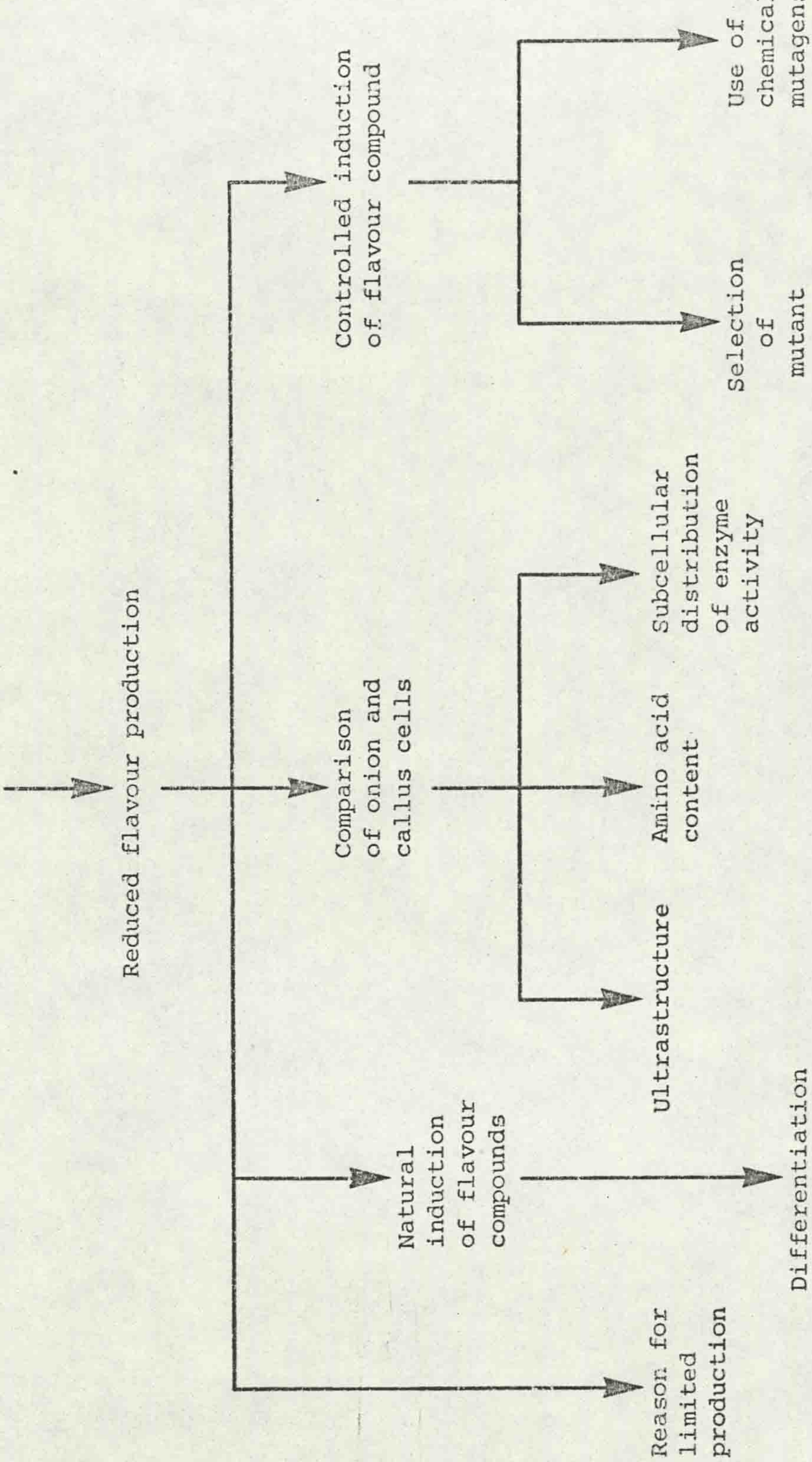
3. Callus tissue was contrasted with tissue from a mature onion bulb to establish any fundamental differences in cell structure or metabolism related to the difference in biosynthetic capacities. A comparison was made of the fine structure and amino acid content of the two cell types and finally, fractionation studies were done to compare the subcellular distributions of alliinase and the flavour

synthesising systems in onion and callus cells.

4. Chemical mutagens were employed to increase the high level of mutation which exists in cultured cells. Mutagen treated tissue was then placed on media containing high levels of flavour compound precursors to select those mutants able to grow and synthesise flavour compounds from the supplied precursors. Similar media were used to select naturally occurring, high yielding clones.

This research programme has been presented in the following flow diagram (Fig. 1.4).

Fig.1.4 Research Programme.



CHAPTER 2

CULTURE OF ONION ROOT CALLUS

2.1 Introduction

Higher plant tissues and cells have been cultured for a relatively short time (Mandels 1972) yet it is now more or less a matter of routine to culture most dicotyledon species. However, continued culture of monocotyledons has long proved difficult, a fact which may be related to the absence of secondary growth in most monocotyledon species (Fisher 1973).

The first monocotyledon cultures were those from storage organs of members of the Araceae family, reported by Morel and Whetmore (1951) where the medium was supplemented with coconut milk and 2,4-D. During the last few years, reports on the successful culture of monocotyledons have become more frequent and it is now possible to grow callus from many species on relatively simple, chemically defined media, e.g. Rye grass (Norstog 1956), maize (Tamaoki and Ullstrup 1958), oats (Webster 1966, Carter 1967), rice(Yamada et al. 1967), wheat (Trione et al. 1968, Schenk and Hildebrandt 1972), Lilium spp. (Sheridan 1968), sugar cane (Nickell and Maretzki 1969) and asparagus (Mullin 1970).

Several workers have reported the isolation and successful culture of tissues derived from onion (Krikorian and Katz 1968, Klein and Edsall 1968, Fridborg 1971, Dunstan and Short 1977) and the closely related garlic (Novak 1974, Kehr and Schaeffer 1976, Abo El-Nil 1977). Most of these studies were concerned with callus growth and morphogenesis, particularly in relation to the production of disease-free cultivars such as in garlic.In contrast, work done

by Davey et al. (1974), Freeman et al. (1974) and Selby and Collin (1976) was directly concerned with flavour production in onion callus cultures. In all the work on flavour production in callus, the cultures were grown on chemically defined media, rather than the non-synthetic media used in earlier studies. In this investigation, onion root callus was also grown on a simple, chemically defined medium to provide cultures in which the amino acid metabolism and flavour production could be examined.

2.2 Materials and Methods

2.2.i Media Preparation

Initial cultures were maintained on B5 medium as developed by Gamborg et al. (1968) and modified by Fridborg (1971) (Table 2.1). After a period of eighteen months of subculture on this medium, cultures were transferred to a new medium developed by Dunstan and Short (1977). This BDS medium was a modified B5 medium with increased levels of phosphate and nitrogen and decreased level of 2,4-D (Table 2.2).

Stock solutions of inorganic salts, vitamins, hormones and Fe.EDTA were prepared in a concentrated form and stored at -20°C until required. The medium was solidified with 1% agar and sterilised by autoclaving at 15psi for fifteen minutes.

2.2.ii Induction and Maintenance of Callus

Seeds of Allium cepa L. cv. Rijnsburger obtained from Asmer Seeds Ltd., Ormskirk, Lancs., were surface sterilised for 40 minutes in a 10% solution of either calcium hypochlorite or 'Domestos', with the addition of a few drops of Teepol (British Drug Houses Ltd.) to aid surface wetting. After several washes in sterile distilled water the seeds were transferred individually to McCartney vials each containing 10 ml agar-solidified nutrient medium. All sterile operations were performed in a laminar air flow cabinet.

After ten weeks incubation in darkness at 26°C seedlings were transferred to 50 ml, wide-necked Erlenmeyer flasks each containing 20 ml solid nutrient medium. Thereafter, callus tissue was subcultured on to fresh medium every six weeks. Only straw-coloured callus was transferred. Any brown material was discarded since this indicated localised cell death which rapidly spread throughout the tissue.

The main problems encountered in the culture of onion callus tissue were its slow growth and the length of time required to build

Table 2.1 Composition of nutrient medium for callus growth.

(Fridborg 1971)

Compound	Weight l^{-1}, (mg)	Molarity, (μM)
$CaCl_2.2H_2O$	150.0	1.02×10^3
KNO_3	3.0×10^3	29.7×10^3
$(NH_4)_2SO_4$	134.0	1.01×10^3
$MgSO_4.7H_2O$	500.0	2.03×10^3
$MnSO_4.4H_2O$	13.2	59.2
$ZnSO_4.7H_2O$	2.0	6.96
$CuSO_4.5H_2O$	0.039	0.155
KI	0.75	4.52
$CoCl_2.6H_2O$	0.025	0.105
H_3BO_3	3.0	48.5
$Na_2MoO_4.2H_2O$	0.25	1.03
$NaH_2PO_4.2H_2O$	169.6	1.09
$FeSO_4.7H_2O$	27.3	100.0
Na_2EDTA	37.2	100.0
Nicotinic acid	1.0	8.13
Thiamine HCl	10.0	29.6
Pyridoxine HCl	1.0	4.86
meso-Inositol	100.0	1.8×10^3
2,4-D	1.1	5.0
Sucrose	20.0×10^3	58.4×10^3
Agar	10.0×10^3	
pH 5.5		

Table 2.2 Composition of improved nutrient medium for callus growth

(Dunstan and Short,1977)

Compound	Weight l^{-1},(mg)	Molarity,(μM)
$CaCl_2.2H_2O$	150.0	1.02×10^3
KNO_3	2.53×10^3	25.02×10^3
NH_4NO_3	320.16	4.0×10^3
$NH_4H_2PO_4$	230.06	2.0×10^3
$(NH_4)_2SO_4$	134.0	1.01×10^3
$MgSO_4.7H_2O$	247.0	1.0×10^3
$MnSO_4.4H_2O$	13.2	45.0
$ZnSO_4.7H_2O$	2.0	6.95
$CuSO_4.5H_2O$	0.039	0.1
KI	0.75	4.52
$CoCl_2.6H_2O$	0.025	0.105
H_3BO_3	3.0	48.5
$NaMoO_4.2H_2O$	0.25	1.03
$NaH_2PO_4.2H_2O$	172.0	1.04
$FeSO_4.7H_2O$	27.85	100.0
Na_2EDTA	37.25	100.0
Nicotinic acid	1.0	8.13
Thiamine HCl	10.0	30.0
Pyridoxine HCl	1.0	4.86
meso-Inositol	100.0	1.8×10^3
2,4-D	0.2765	1.25
Sucrose	30.0×10^3	88.0×10^3
Agar	10.0×10^3	
pH 5.5		

up stock cultures from seed. Another problem was the periodic depletion of stock cultures which occurred when the callus became contaminated.

2.2.iii Contamination of Stock Cultures

During the period when the level of contamination was high, contaminated tissue showed a decline in growth. It also became very soft, friable and brown in colour, and a white film of bacteria rapidly covered the surface of the agar. As reported by Winton and Mathes (1973) for aspen callus cultures, a white callus indicates rapidly growing cells while a decrease in growth is accompanied by an increase in brown pigmentation, so the presence of contamination could be easily detected by early browning of the tissue. The bacterial contamination is believed to be inherent in the callus and it only became apparent when tissue growth declined at the end of a passage. Similar findings were reported by Trione et al. (1968) who observed that young callus cultures which appeared to be free of micro-organisms suddenly showed bacterial contamination. Similarly Schenk and Hildebrandt (1972) reported that many seeds have a high percentage of internal microbial contamination. In the case of the onion callus, bacterial contamination was more frequent in old callus stocks that had been subcultured for several years.

Some of the newly initiated callus showed a tendency to red pigmentation which was identified by Selby and Collin (1976) as being due to contamination by a motile bacillus. Callus initiated on BDS medium rarely exhibited this pigmentation. To prevent the spread of contamination in the callus stocks, during routine subculture, all flasks containing brown or reddish tissue were discarded.

2.2.iv Growth Analysis

A growth analysis of the callus during one passage was carried out to establish the stage of callus growth most appropriate for use in metabolic studies. The analysis was continued over a nine week period, with a weekly sampling of ten 50ml flasks each containing 20ml solid nutrient medium and an initial inoculum fresh weight of approximately 1.5g. The fresh weight of callus was measured, then the tissue dried at 80°C for two days and re-weighed to obtain dry weight data.

2.2.v Induction of Morphogenesis

Following the method of Thomas and Davey (1975), callus was transferred to medium lacking the growth hormone, 2,4-D, and incubated in darkness to initiate root production.

To initiate shoots, callus which had only been cultured for a maximum of six months was transferred to media in which the 2,4-D was replaced by NAA and a cytokinin, 2iP, was added. The levels of growth hormone used were 2 mg l^{-1} NAA and 8mg l^{-1} 2iP (Dunstan 1977), 0.5mg l^{-1} NAA and 2mg l^{-1} 2iP, and 1mg l^{-1} 2iP. Flasks were either incubated in a 16 hour photoperiod immediately after subculture or left in darkness for three weeks before transferring to the light.

2.3 Results and Discussion

2.3.i Induction of Callus Formation

Ten weeks after the seeds were sown on agar only 50% of them had germinated (Fig. 2.1). After germination, the root cells proliferated to form a callus mass of undifferentiated cells whilst the shoot quickly shrivelled and died (Plate 2.1). The tissue grew as a compact, straw-coloured nodular callus with mucilage on the surface producing a glistening appearance (Plate 2.2a). With long term subculture the morphology of the callus changed to a soft, dry and very friable tissue with a crystalline appearance (Plate 2.2b). Even when grown in the light, callus tissue was devoid of chlorophyll except in regenerated shoots or shoot initials.

2.3.ii Effect of Media on Callus Growth

Cultures were transferred to BDS medium since it was reported by Dunstan and Short (1977) to produce a forty-fold increase in tissue fresh weight over an eight week subculture period. This is in comparison with a two to three fold increase over a six week passage on the modified B5 medium. The callus grew much faster on the BDS medium although only a four to five fold increase in fresh weight was recorded.

2.3.iii Growth Analysis

Figures 2.2a and 2.2b show that the callus exhibits a typical sigmoidal growth pattern, with a short lag

Fig. 2.1 % germination of surface sterilised onion seeds on nutrient agar.

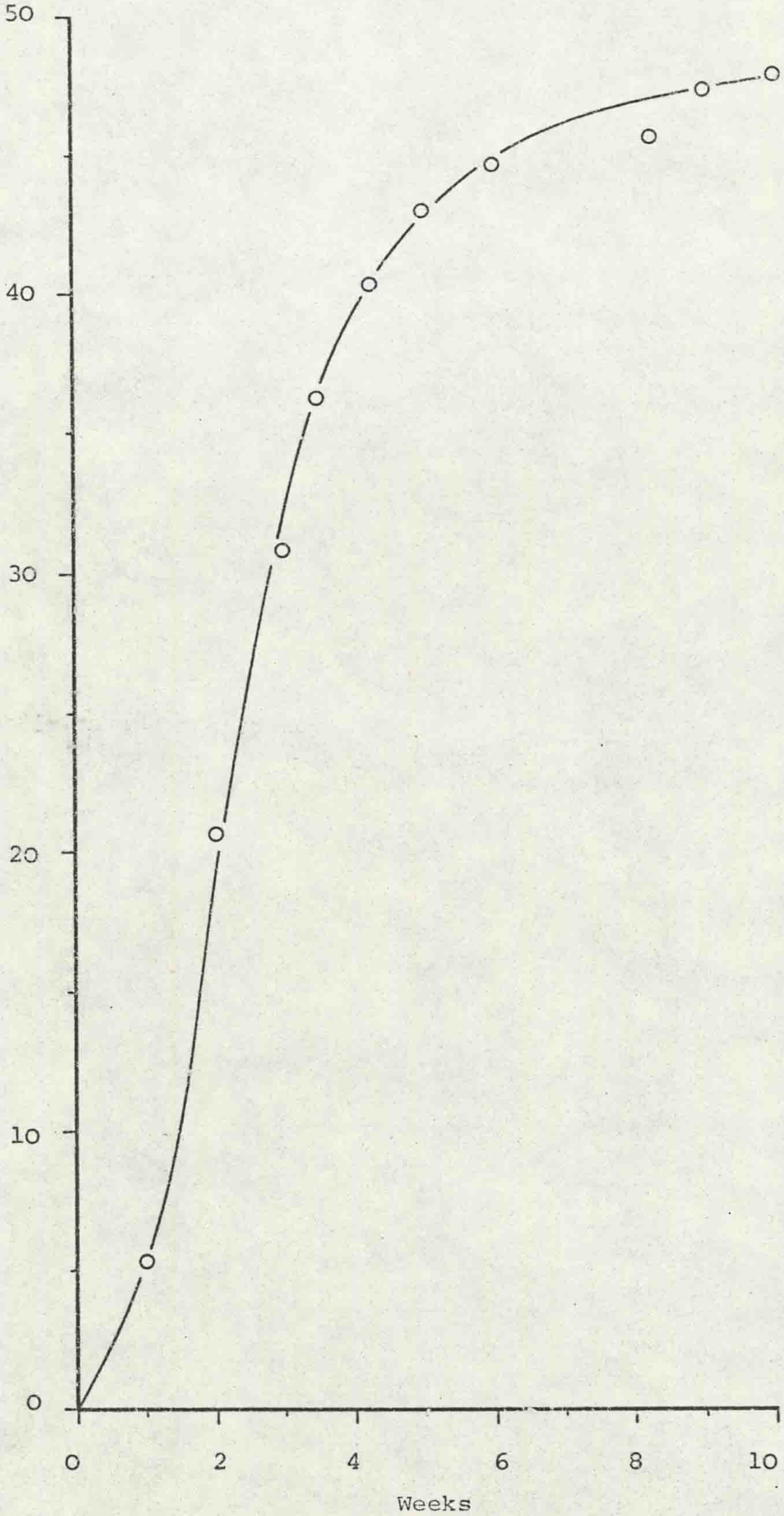

50
40
30
20
10
0
% Germination
0
2
4
6
8
10
Weeks

Plate 2.1 Stages of onion callus initiation showing callus developing from the root.

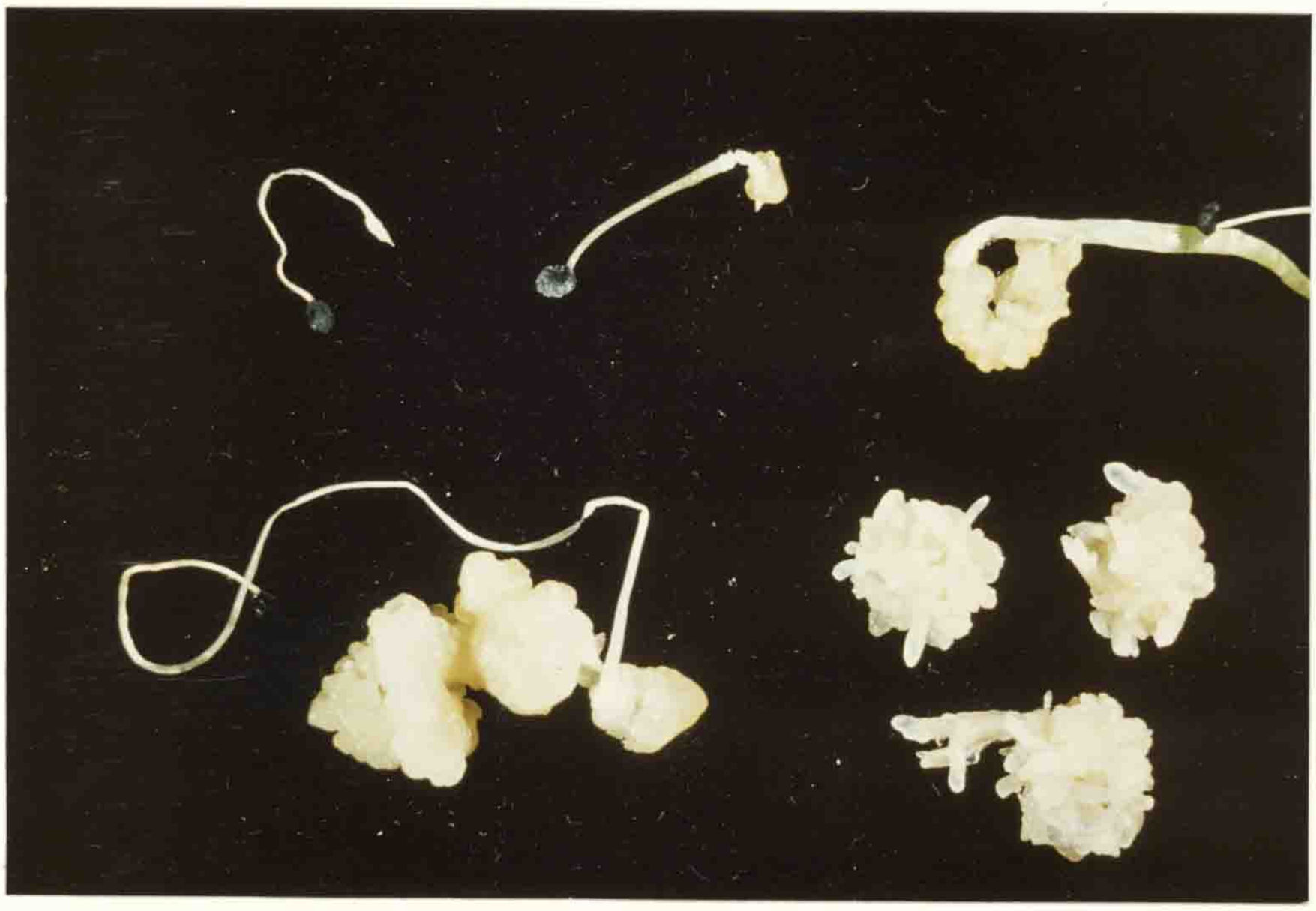


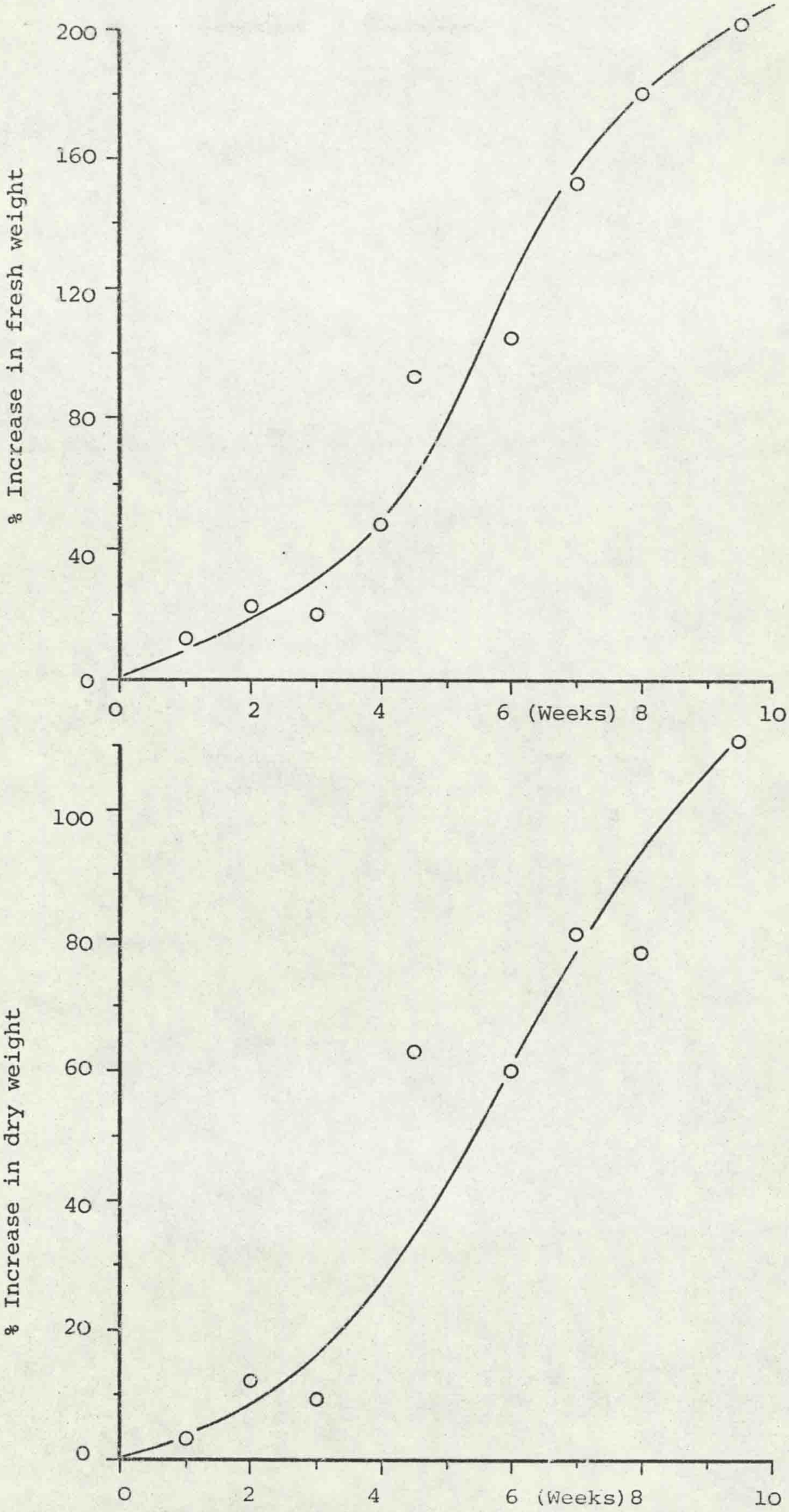
Plate 2.2 Appearance of onion root callus, a) newly initiated, compact, nodular callus with a surface covering of mucilage, b) soft, friable callus after several months in culture.

a

b

Fig. 2.2 % increase in fresh weight (a) and dry weight (b) of onion callus during one subculture of nine weeks.

a


% Increase in fresh weight
200
160
120
80
40
0
0
2
4
6 (Weeks)
8
10

b

% Increase in dry weight
100
80
60
40
20
0
0
2
4
6 (Weeks)
8
10

phase followed by a period of rapid growth. Even after nine weeks, fresh and dry weights were still increasing.

Callus tissue was normally subcultured every six weeks, so in this time growth was never being limited by depletion of nutrients from the medium. From these results, it would be possible to sample at any time during the growth cycle except in the first week after subculture.

The average water content of callus tissue was 95% compared with 86% for onion bulb cells. This is because actively dividing cells absorb more salts and water (Yeoman 1970) than do differentiated cells. Hence, cultured cells tend to have a higher water content than their counterparts in the intact plant.

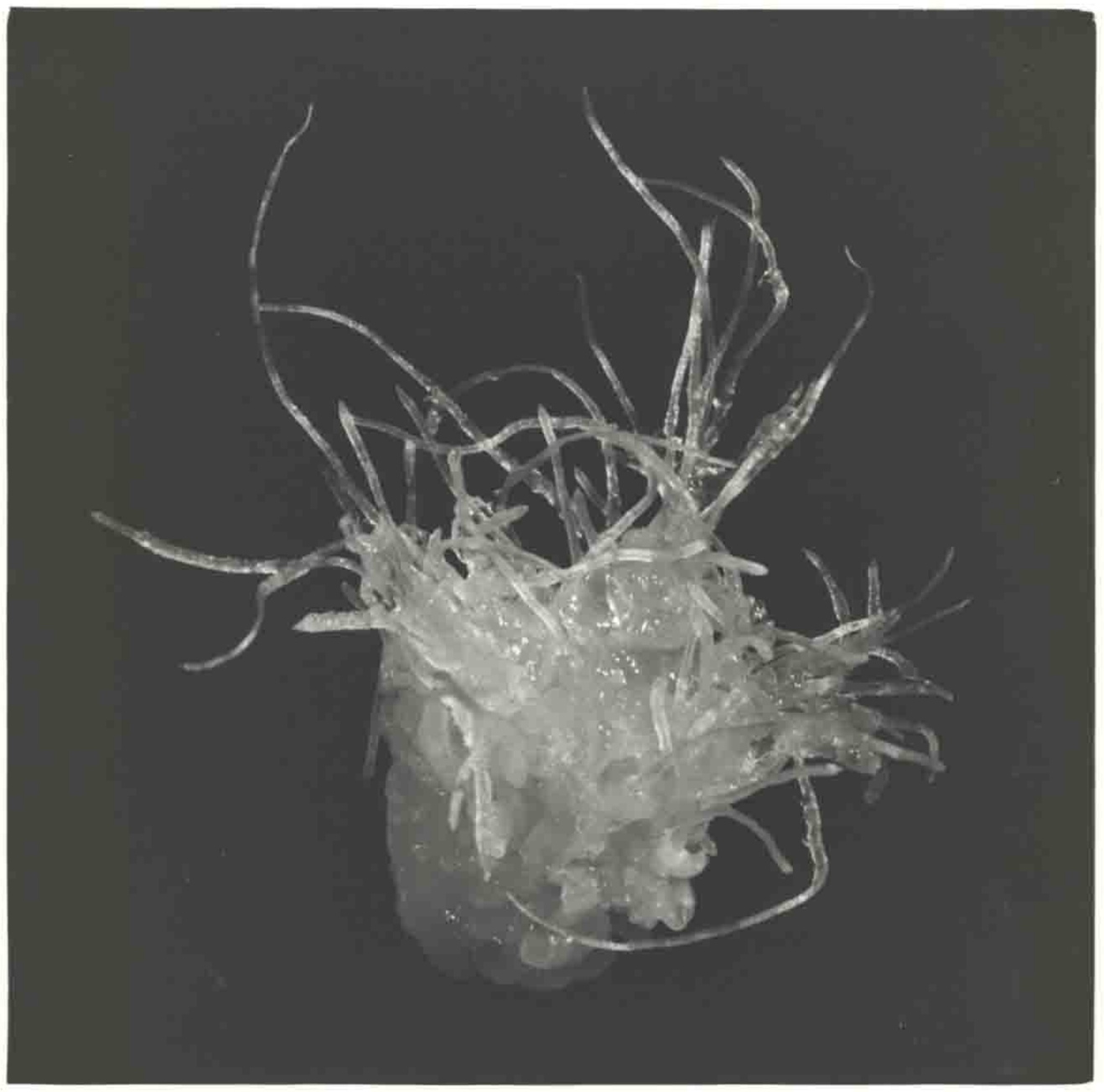
2.3.iv Induction of Morphogenesis

Within two weeks of transfer to medium lacking 2,4-D, large numbers of normal roots had formed from the callus (Plate 2.3). Most roots grew into the agar although some grew aerially from the upper portion of the callus. Callus which had been cultured for several years also produced roots when placed on the appropriate medium but in this case the roots formed much more slowly.

Only newly initiated callus was used for shoot formation since after about six months callus tissue rapidly lost the ability to regenerate shoots (Thomas and Davey 1975). No recognisable shoots were formed from

Plate 2.3 Root production from onion callus growing on nutrient medium in the absence of 2,4-D.

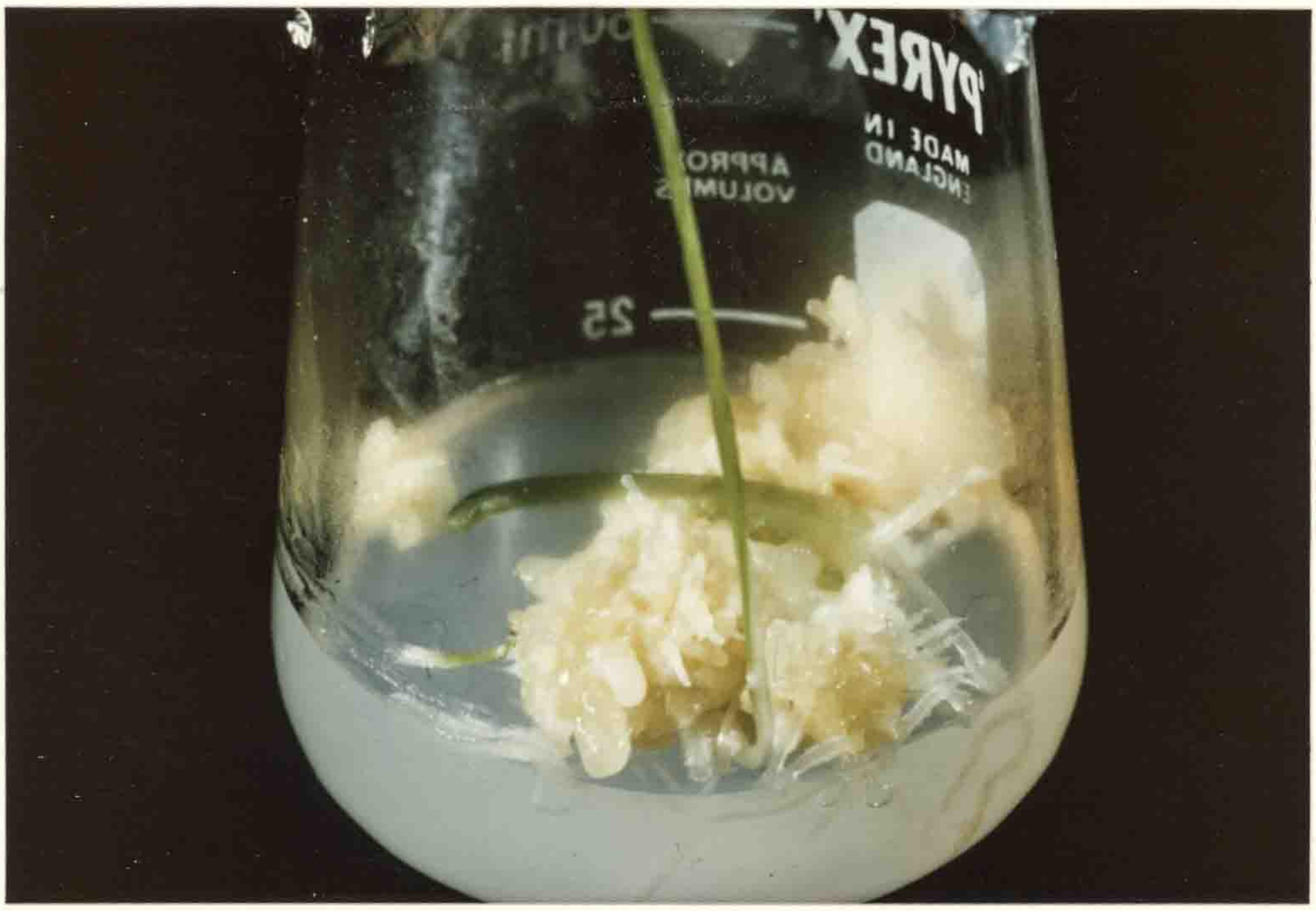

APPROX:
VOLUMES



callus on the shoot inducing medium, the only differentiation observed being in the form of thick, woody, root-like structures which occasionally produced chlorophyll. These have also been reported by Fridborg (1971). True onion shoots were produced from several clones on media lacking 2,4-D. As soon as the shoots were detected, which was approximately three weeks after subculture, the flasks were transferred to the light where the callus continued to produce many shoots (Plate 2.4a) and green areas. Some of these shoots could be separated from the callus as plantlets which were transferred first of all to agar (Plate 2.4b) then to pots containing a sterile mixture of John Innes Potting Compost No.2 and acid washed sand. Although the plantlets were covered with jam jars to reduce transpiration, most of them died due to excessive water loss. Callus produced plantlets had poorly developed root systems and this seemed to be the reason for their failure to grow successfully in soil. Normal onion seedlings also suffered from desiccation for the same reasons when they were kept under greenhouse conditions.

Plate 2.4 Shoots regenerated from onion callus growing on agar in the absence of 2,4-D, a) in association with the callus mass, b) plantlet removed from the callus to agar prior to planting in sterile compost.

a



b

CHAPTER 3

BIOSYNTHESIS OF S-PROPENYL L-CYSTEINE SULPHOXIDE IN CALLUS CULTURES.

3.1 Introduction

Few studies have been carried out on the biosynthesis of flavour compounds in onion and garlic tissues. In 1962, Suzuki et al. reported the incorporation of radioactively labelled valine into S-(2-Carboxypropyl)-L-cysteine (CPC) and S-(2-Carboxypropyl)-glutathione (CPG) in excised root cultures of garlic. It should be noted that all amino acids are of the L-configuration. They suggested that the origin of the 2-carboxypropyl group was methacrylic acid or methacrylyl CoA which were known to be intermediates of valine metabolism. This conversion of valine to methacrylic acid was known to operate in animal systems and although CPC had been isolated from animal fluids, the report by Suzuki et al. (1962) was the first record of this compound in plant tissues.

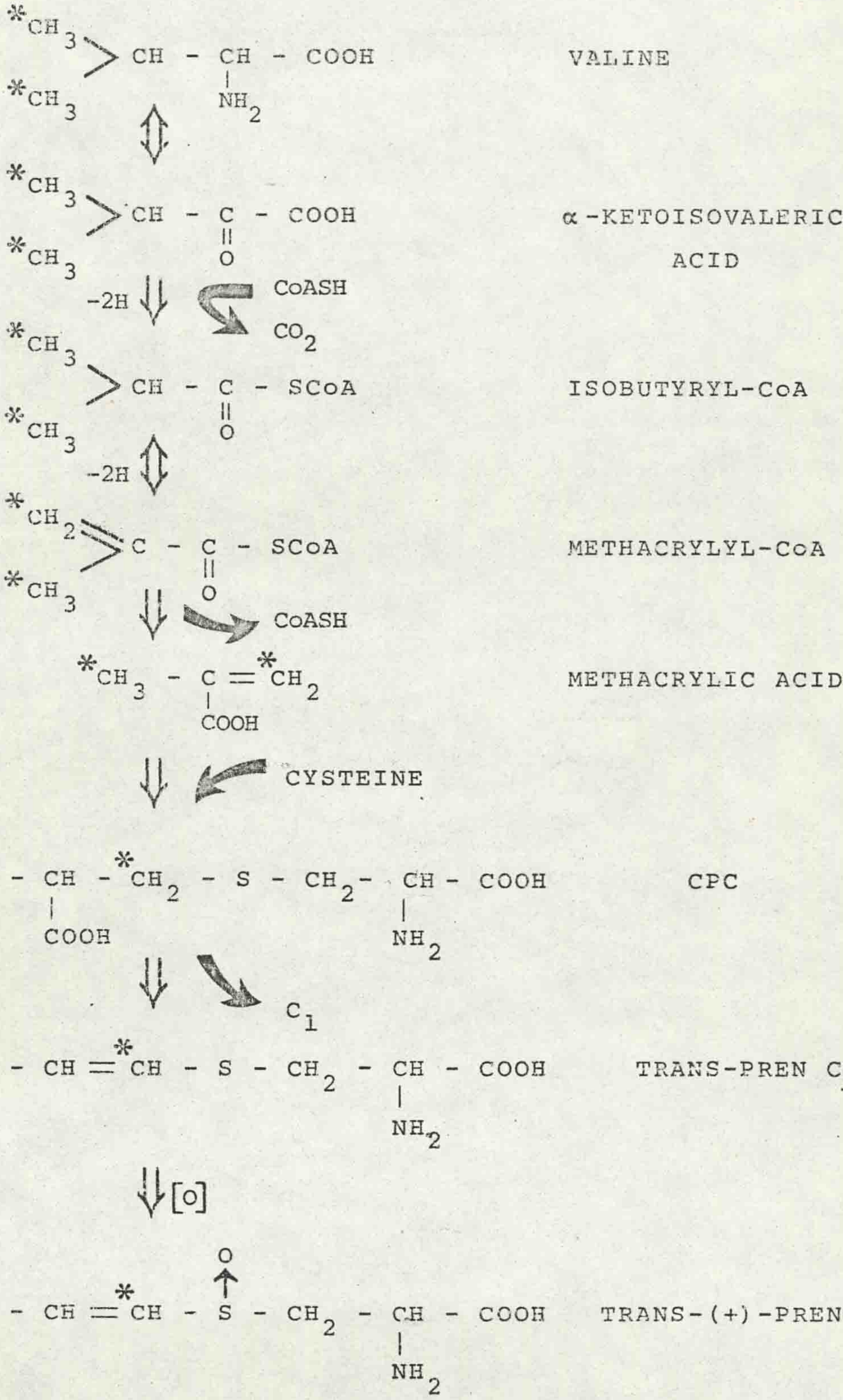
Similar experiments were carried out by Ettala and Virtanen (1962) in which labelled sulphate was injected into onion sets. The plants were sampled at various stages of growth up to 85 days after injection when the onion had produced green leaves. Seven days after injection, 21 radioactive spots were detected on a paper chromatogram of the amino acid fraction prepared from the plant. Included amongst the radioactive compounds were cycloalliin, γ-glutamyl carboxypropyl cysteine and γ-glutamyl (+)-S-propenyl-L-cysteine. By injection of methacrylic acid into sprouting bulbs and subsequent isolation of the resulting free CPC with the naturally-occurring bound

form of CPC , Granroth and Virtanen (1967) were able to confirm that CPC is formed by the reaction of cysteine with methacrylic acid. Injection of ^{14}C-cysteine into sprouting bulbs led to the labelling of CPC, although it was often present at low levels which were not detectable with ninhydrin. When ^{14}C-CPC was injected into the sprouting bulbs, 40-50% of the administered dose was metabolised in one day and up to 97% was metabolised by the end of one week. The main product was cycloalliin which accounted for 17% of the label after seven days although some label was found in Pren Cy SO. It was concluded that Pren Cy SO was the end product of the biosynthetic chain and, as such, metabolically inert. As a result of this work, Granroth and Virtanen proposed the formation of CPC from methacrylic acid and cysteine and the further conversion of CPC to the end product, Pren Cy SO which was found in large amounts in onion tissues.

This work was continued so that in 1970, Granroth proposed the pathway for the synthesis of Pren Cy SO as shown in Fig. 3.1. This shows clearly how the label from ^{14}C-valine was incorporated into each of the intermediates of the pathway leading to the production of Pren Cy SO. Thus, it was demonstrated that the propenyl portion of Pren Cy SO was derived from valine. Further confirmation of the pathway was obtained by feeding ^{14}C-α-ketoisovaleric acid to onion. However, several points still require clarification. For example, further work is needed on the enzymes involved in Pren Cy SO synthesis.

Fig. 3.1 Pathway of biosynthesis of trans-(+)-S-(propenyl)-L-cysteine sulphoxide from valine and cysteine as proposed by Granroth (1970).

* ^{14}C label.


VALINE
α-KETOISOVALERIC ACID
CoASH
CO2
ISOBUTYRYL-CoA
METHACRYLYL-CoA
CoASH
METHACRYLIC ACID
CYSTEINE
CPC
C1
TRANS-PREN CYS
[O]
TRANS-(+)-PREN CY SO

Since the biosynthesis of onion flavour compounds was now well established, interest was directed to the possibility of producing flavour compounds in cultured cells and tissues derived from the onion plant. However, it was shown by Davey et al. (1974) and Freeman et al. (1974) that onion callus tissue failed to produce significant amounts of the characteristic flavour compounds. Similar findings were reported by Selby and Collin (1976) where it was estimated that the level of flavour compounds in onion callus was only 10% of the level in the whole plant. Personal communication with Selby and Collin also suggested that the low level of flavour compounds in the callus was due to the three [redacted] cysteine sulphoxides and that the important flavour precursor of onion, Pren Cy SO, was absent from the callus.

Since Pren Cy SO was not detected in callus tissue by TLC-electrophoresis, it seemed reasonable to assume that either the end product was synthesised and immediately removed, perhaps by degradation or formation of complexes with other molecules, or that the biosynthetic pathway leading to its formation was not operating as normal. This may be due to the inhibition or absence of one or more of the enzymes involved in the pathway.

The aim of this investigation was to study the biosynthetic pathway of Pren Cy SO in onion and callus tissues in an attempt to determine the reason for the lack of this flavour precursor compound in callus. The investigation took the form of feeding

experiments which were carried out in three separate stages:

a) the feeding of physiologically high levels of pathway intermediates

b) the use of radioactive tracers

c) the feeding of the cysteine derivatives involved in the pathway.

Earlier work by Selby (Unpublished results) was concerned with the feeding of low levels of intermediates to callus tissue. Sodium sulphate, valine, methacrylic acid, cysteine and CPC were added to the medium at levels up to 3mM and tissue extracts analysed by TLC-electrophoresis. Results of these experiments indicated that the biosynthetic pathway for production of Pren Cy SO may be blocked at the point of CPC synthesis, since it was observed that feeding of intermediates before this step did not result in Pren Cy SO formation, although this compound was in fact produced when CPC was fed to callus.

It is now widely accepted that cells and tissues in culture should be capable of producing the secondary products characteristic of the intact plant. Therefore, by appropriate control of growth conditions and availability of biosynthetic precursors it should be possible to induce the synthesis of secondary metabolites and control the yield of the desired compounds. Thus, it was considered appropriate in this investigation to feed physiologically high levels of intermediates to the callus in an attempt to induce synthesis of Pren Cy SO. The use of this approach is exemplified

by the following examples in which the yield of metabolites was increased by feeding precursors to the tissue. These include the feeding of phenylalanine to suspension cultures of Datura stramonium as an intermediate in the synthesis of alkaloids (Furuya 1968), the use of cholesterol as a key intermediate of diosgenin synthesis in Dioscorea deltoidea callus cultures (Kaul et al. 1969) and the stimulation of alkaloid production by tryptophan in callus cultures of Peganum harmala (Nettleship and Slaytor 1974).

In the investigation reported here, the intermediates were fed to the callus to try and induce activation and/or synthesis of the enzyme which catalyses the conversion of cysteine and methacrylic acid to CPC.

In order to examine the biosynthetic pathway in detail, radioactive tracers were used. Thus it was possible to follow the label as it was metabolised and also to detect compounds which were present in trace amounts and therefore not detectable by the ninhydrin method. This experiment also provided a suitable method of feeding cysteine to callus, since feeding of high levels was difficult.

Further experiments were carried out in which low levels of the cysteine derivatives, CPC, Pren Cys and Pren Cy SO were applied to callus. These experiments were designed to test the operation of the latter part of the biosynthetic pathway from CPC to Pren Cy SO and to determine the response of callus tissue to exogenously supplied Pren Cy SO.

3.2 Materials and Methods

3.2i High Level Feeding of Intermediates

Sodium sulphate, valine and methacrylic acid were added individually to standard nutrient medium before autoclaving at six concentrations, 0mM, 10mM, 20mM, 50mM, 70mM and 100mM, and callus transferred directly onto these media at the time of routine subculture. All feeding experiments were done using McCartney vials each containing 10ml nutrient agar.

Tissue growth was assessed at the end of each six week passage by fresh weight measurements and the results expressed as percentage increase in fresh weight over each passage. Ten replicates were used at each sample time.

Production of flavour compounds was detected by crushing the callus tissue and noting the presence or absence of the onion smell. This provided a rapid screening method for production of flavour compounds by callus since the onion smell is readily detectable at very low levels. Since the effect of treatment with high levels of intermediates is likely to be a long term effect, selected treatments were maintained after the first passage.

Attempts were made to incorporate high levels of cysteine, cysteine hydrochloride or cysteic acid into the medium but in each case several problems arose. The amino acids were not readily soluble in the medium at high concentrations and on autoclaving they were oxidised or

otherwise degraded. This degradation resulted in a drop in pH which subsequently prevented the agar from setting. Thus, it was decided to use a very low level of radio-actively labelled cysteine. This experiment is reported in Section 3.2ii.

CPC was synthesised (Appendix 1.vii) and fed to callus via the medium at low levels of 4, 8, 12, 16 and 20mM then at higher levels of 30mM and 100mM. Tissue on low CPC levels was transferred to successively higher levels over four passages. Growth response and flavour production were recorded as described and tissue stored at -20°C for analysis.

3.2ii Feeding of Radioactive Isotopes

Callus Tissue

A total of 0.3μ Ci (60μ l) cysteine hydrochloride, 0.5μ Ci (20μ l) valine or 0.5μ Ci (10μ l) serine was placed on top of a small piece of callus of known fresh weight (approximately 100mg). The tracers were all uniformly labelled with ^{14}C and were obtained from The Radiochemical Centre Ltd. The radioactive compounds were dissolved in sterile distilled water and dispensed in 10 or 20μ l aliquots using an automatic pipette (Sampler Micro-Pipetting System, Oxford Laboratories International Corporation) with sterile dispenser tips. Tissue was sampled after 3, 7 and 14 days for amino acid analysis by TLC-electrophoresis and autoradiography. Methods are described in detail in Appendix 2. Controls comprised of equal volumes of sterile distilled water placed on top of the callus.

Onion bulb slices

Thin slices of tissue of known fresh weight (approximately 100mg) from the centre of a dormant bulb were placed on nutrient agar and 0.3 μ Ci cysteine hydrochloride or 0.5 μ Ci valine were added as described above. The material was sampled after three days incubation and the extract analysed by TLC-electrophoresis and autoradiography.

Onion Shoot Tips

Onion bulbs were placed in the necks of small jam jars containing water, care being taken not to wet any part of the bulb as this caused rotting of the tissue. The bulbs were incubated in a 16 hour light regime until roots and leaves were produced.

The tips were cut from the first emergent leaves when they were a few centimetres long, each tip being approximately 100mg fresh weight and 2.5cm long. The cut surface of the tip was rinsed with distilled water and carefully blotted dry, then allowed to take up 0.5μ Ci label from a small glass tube. Compounds fed were uniformly labelled ^{14}C - cysteine hydrochloride, ^{14}C - valine and ^{14}C- serine. When the tube was dry, the tip was allowed to take up two 200μ l aliquots of distilled water. The vessel was thereafter kept filled with water and the shoot material sampled for analysis after two and four days. Extracts were analysed by TLC-electrophoresis and autoradiography.

3.2iii Preliminary Investigation of Radiochromatograms

TLC plates were examined in a radiochromatogram spark chamber (Birchover Instruments Ltd.) before autoradiography was carried out. This confirmed that radioactivity had been incorporated into the sampled tissue. However, the method could only be used in a preliminary investigation since it is not sensitive enough to enable identification of all the radioactive compounds present.

3.2iv Autoradiography

The TLC plate was left in contact with X-ray film (Ilford 'Ilfex') for two weeks then the films developed as described. To ensure good contact between the thin layer and the film, a glass plate was placed on top of the film as a weight with a piece of paper between the film and the glass to prevent damage to the film surface.

The films were developed for 4 minutes in DX80 developer (Kodak) diluted 1 in 4 with water, placed in a stop bath for 15 seconds then fixed for 10 minutes. After washing in a water bath for 30 minutes, the films were dipped into wetting agent and allowed to dry. All operations were carried out using a red safe light.

3.2v Feeding of Cysteine Derivatives.

60 µl 50 mM CPC solution, 100 µl 5mM Pren Cys solution or 100 µl 5mM Pren Cy SO solution were placed on

top of a small piece of callus of known fresh weight (approximately 100mg) as described in section 3.2ii. The tissue was incubated for 3, 7 and 14 days before sampling for analysis as described in Appendix 2. At each sampling time, two extracts were prepared, one being examined by TLC-electrophoresis and the other by amino acid analysis.

CPC was dissolved in sterile distilled water and dispensed under sterile conditions. Pren Cys and Pren Cy SO are sparingly soluble in water so more dilute solutions were prepared and then filter sterilised before use using a Millipore filter. CPC, Pren Cys and Pren Cy SO were synthesised as described in Appendix 1.vii, 1.v and 1.vi respectively.

The method of applying the test solution directly onto the callus provided a simple and rapid experimental system using the minimum amounts of plant material and test compounds. It enabled sampling to be carried out after only a few days, compared with a period of weeks when material was fed via the medium, and it eliminated the risk of compound degradation on autoclaving. The main disadvantage was the extra risk of contamination due to increased handling of the tissue, but in all of the experiments reported here, no contamination was observed.

3.2vi Amino Acid Analysis

Dried extracts, each equivalent to approximately 100mg fresh weight of tissue were stored at -20°C before analysis.

Amino acid analyses were carried out using a Jeol JLC-6AH amino acid analyser. Peak areas and retention times were recorded using an integrator. Often visual inspection of the chromatography trace was more useful as comparative studies were particularly important in this work. The sample for amino acid analysis was applied tc the analyser in pH2.2 citrate buffer.

3.3 Results

3.3i High Level Feeding of Intermediates

Treatment with methacrylic acid at all levels prevented callus growth (Table 3.1) and the tissue rapidly showed evidence of senescence.

Some growth occurred at all levels of sulphate but growth declined with increasing sulphate concentration (Table 3.1). In all cases, growth was lower than that recorded for the control.

As the level of valine in the medium increased, the percentage increase in fresh weight declined (Table 3.1). The tissue was able to grow very slowly at the lower levels of 10mM and 20mM but growth was prevented at higher levels.

Concentrations of CPC up to 20mM had little detrimental effect on callus growth and values for all treatments up to this level approached that of the control. However, growth was much reduced at levels of 30mM CPC and at 100mM CPC, tissue death occurred. Similarly as callus was transferred from a particular level of CPC to a higher one, growth declined, until, at 100mM the callus died. This decline in growth indicated that there was a threshold level of CPC on which the tissue would grow and that above this level, cell death occurred rapidly.

Occasionally, when callus was placed on CPC-containing

Table 3.1 Effect of feeding high levels of pathway intermediates of Pren Cy SC synthesis on callus growth.

Concentration (mM)	Percentage increase in fresh weight			
	Sodium Sulphate	Valine	Methacrylic acid	CPC
0	275.70	275.70	275.70	108.43
10	211.52	32.60	-15.40	
20	189.92	7.88	-16.01	96.65
30				20.87
50	78.83	-9.85	-20.54	
70	52.61	-10.47	-18.56	
100	26.28	2.03	-15.75	-6.91

medium, the callus surface was found to be covered with white flakes of an unknown compound. Since CPC treatment resulted in the production of flavour precursors as determined by the onion smell which resulted when the treated callus was crushed, this compound may have been Pren Cy SO, but no analysis was carried out.

The growth measurements gave an indication of the extent to which the callus was able to metabolise each intermediate. The tissues which died were presumably unable to metabolise high levels of intermediate whilst those cultures that survived were considered able to utilise these compounds. However, the test compounds also take part in other metabolic pathways and so the ability of a particular culture to survive on the test media did not necessarily mean that the tissue was able to operate the biosynthetic pathway leading to Pren Cy SO production.

No characteristic onion odour was detected when callus tissue from the first three feeding experiments was crushed. However, presence of CPC in the medium at all levels did induce flavour production as determined by the onion smell which resulted on tissue damage. An increase in flavour strength with increasing CPC concentration was not detected at the lower levels of CPC using sensory tests alone. However, amino acid analysis could be employed to provide such information although this was not possible at the time of the experiment. These preliminary results show that whilst valine, sodium sulphate and methacrylic acid do not result in flavour production in callus,

CPC is successfully converted to Pren Cy SO.

Thus, when the tissue is damaged, the newly formed flavour precursor is degraded by the enzyme, alliinase, to release the volatile components of onion flavour and odour. The results described here support the hypothesis of Selby (Unpublished results) which suggested that the biosynthetic pathway of Pren Cy SO in callus tissue was blocked at a particular point, the point of CPC synthesis. This 'block' was most likely to be due to a single enzyme deficiency at the point of CPC synthesis since the pathway from valine to methacrylic acid is part of normal metabolism and is therefore unlikely to be absent from callus, while the latter part of the pathway from CPC to Pren Cy SO has been shown to be operative in callus tissue.

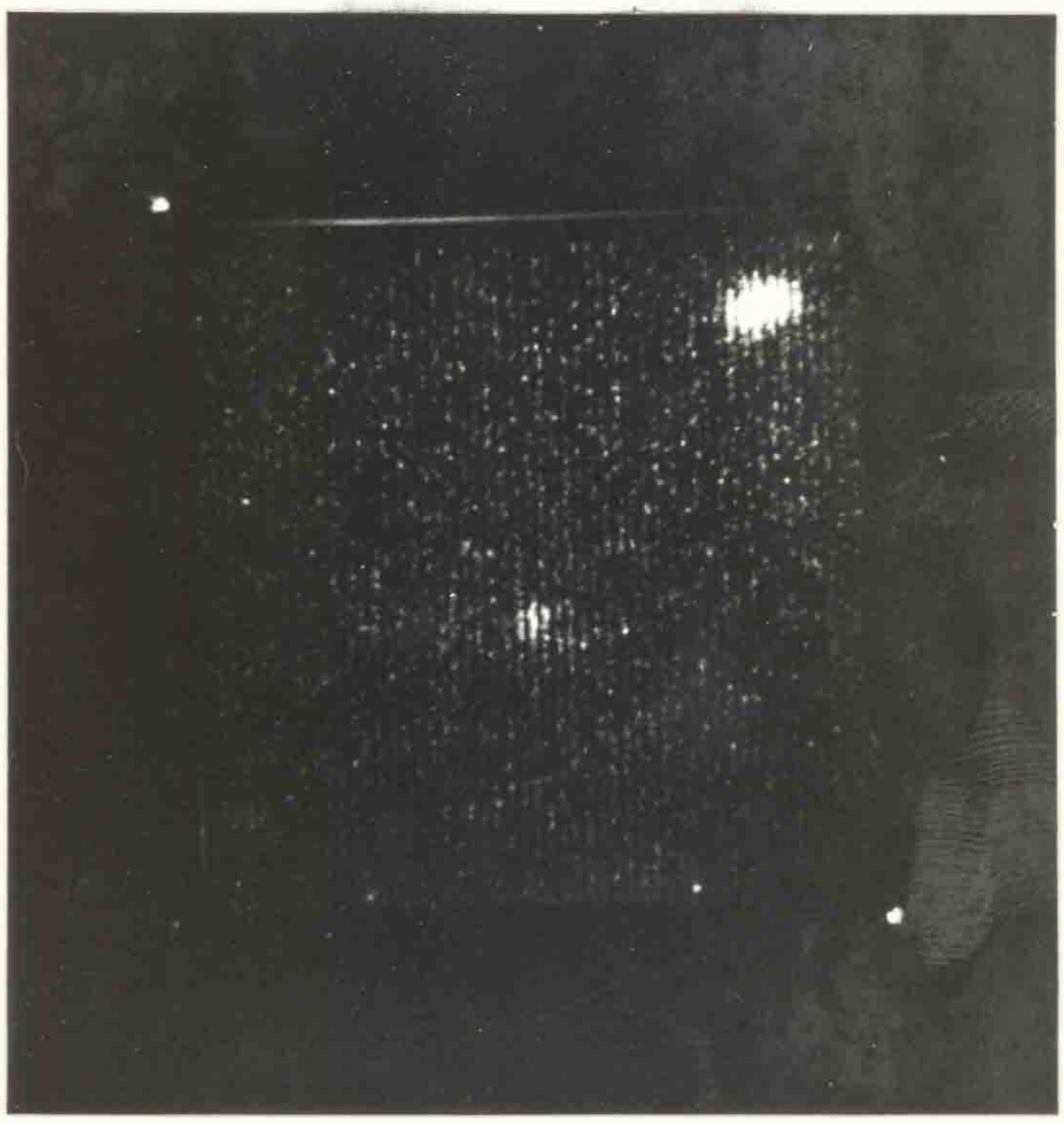
It was noted that the levels of intermediates used in the experiments reported here were extremely high and may have resulted in substrate inhibition of the enzymes under examination. Conversely, the test compounds were incorporated into the medium and so it is not known exactly how much of each intermediate actually entered the cells.

3.3ii Use of Radioactive Isotopes

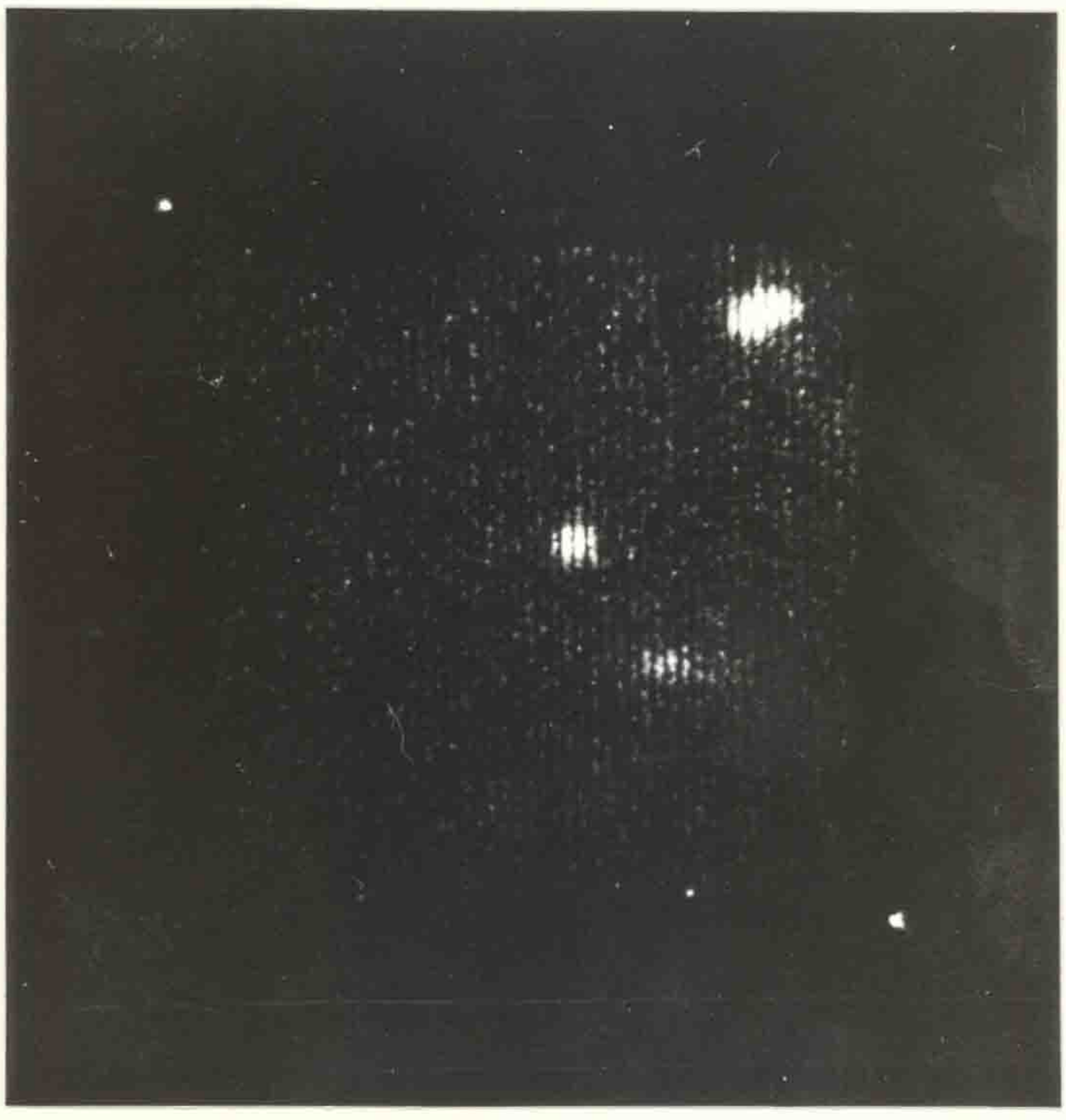
Preliminary examination of radiochromatograms showed that some incorporation of label had occurred, but, as can be seen from plates 3.1a and 3.1b this only gives a rough estimate of actual incorporation. Autoradiography was essential for identification of labelled compounds. However, the spark chamber provided a very useful and rapid method of screening the plates before autoradiography was carried out.

Plate 3.1 Spark chamber photograph of radioactivity emitted from thin layer chromatograms of extracts of callus tissue supplied with a) ^{14}C-cysteine, b) ^{14}C-valine.

a



b



For comparison with radioactively labelled tissues, Plates 3.2a and 3.2b and Figs. 3.2a and 3.2b show the distribution of free amino acids and flavour compounds in extracts of onion bulb and callus tissues respectively. Fig. 3.3 shows a map of the distribution of these ninhydrin positive compounds. Tables showing the labelling of amino acids and flavour compounds and autoradiographs of extracts from callus, onion shoot tips and bulb slices fed with radioactively labelled cysteine, serine and valine are presented in Appendix 3.

After three days in the presence of ^{14}C -cysteine, callus extracts showed labelling of several amino acids (Fig. 3.4a). Those compounds labelled were alanine, glutamine, glutamic acid and Me Cys with heavier labelling of the Me Cy SO, CPC, Pren Cy SO and peptides. A similar pattern was observed after seven and fourteen days with labelling of asparagine and aspartic acid as well as those listed above. Only Me Cy SO, Pren Cy SO and peptides were heavily labelled.

Preliminary experiments, where sampling was done after one, two and three days, showed moderate labelling of Pren Cy SO and this became heavily labelled after three or more days. In all cases, Me Cy SO and peptides were the most heavily labelled.

These results indicate that cysteine is rapidly metabolised in callus tissues to produce Me Cy SO, Pren Cy SO and peptides of unknown composition. The labelling of CPC suggests that cysteine is metabolised by the biosynthetic pathway to the end product Pren Cy SO.

Plate 3.2 Thin layer chromatogram of extracts from a) onion bulb tissue, b) onion callus tissue.

a

b

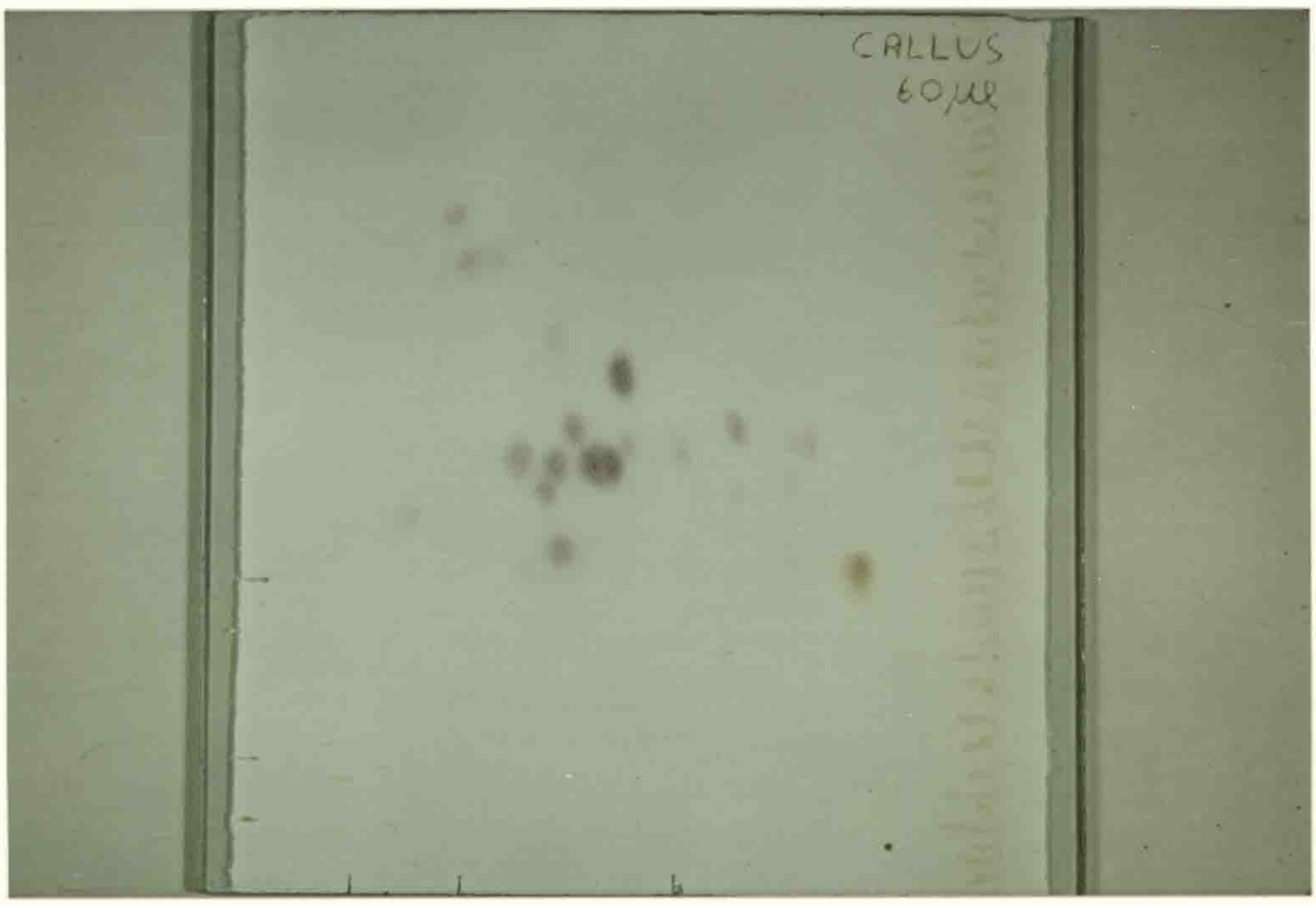
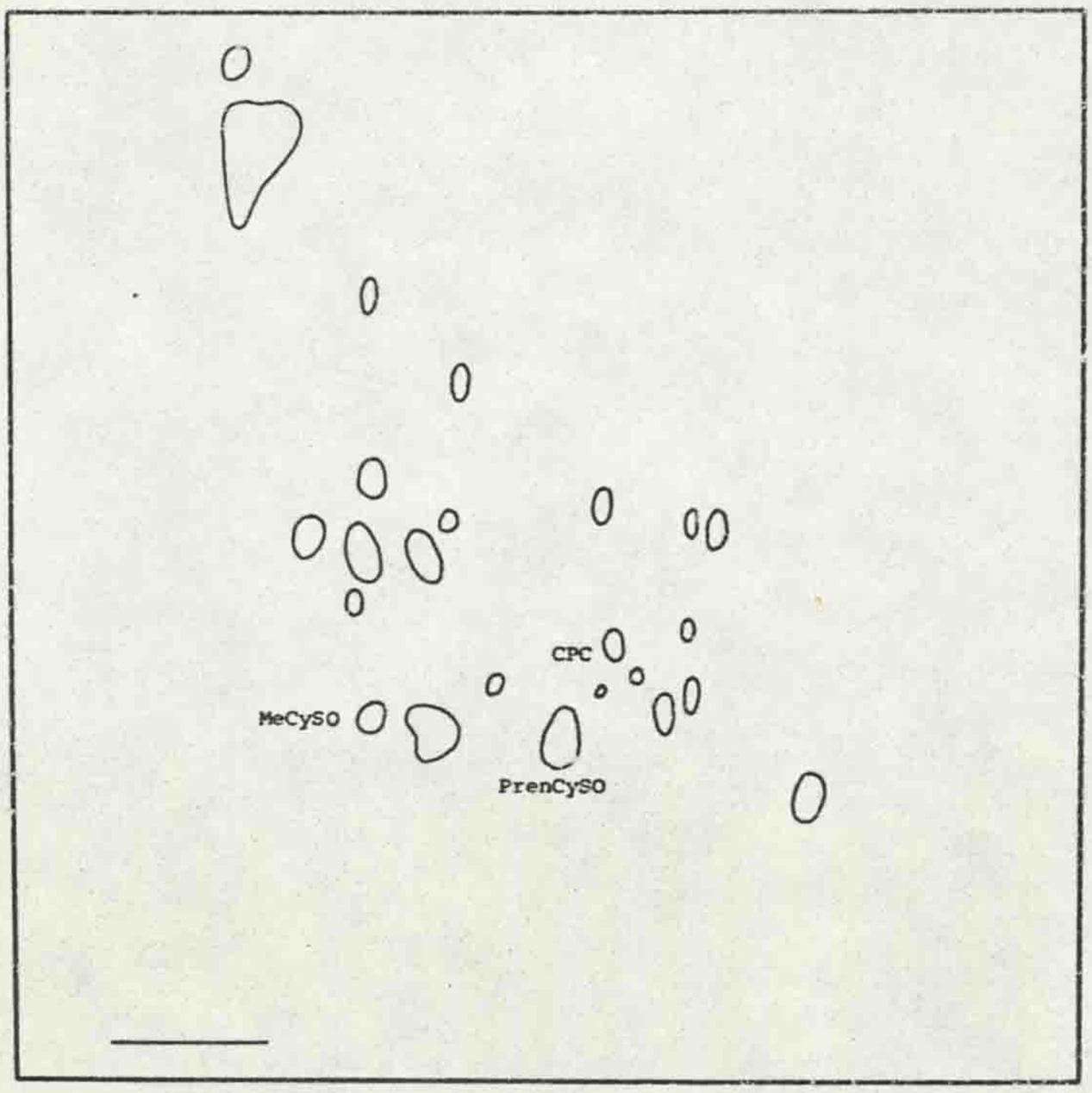


Fig. 3.2 Thin layer chromatogram of amino acid extracts of a) onion bulb tissue, b) onion callus material.

a



b

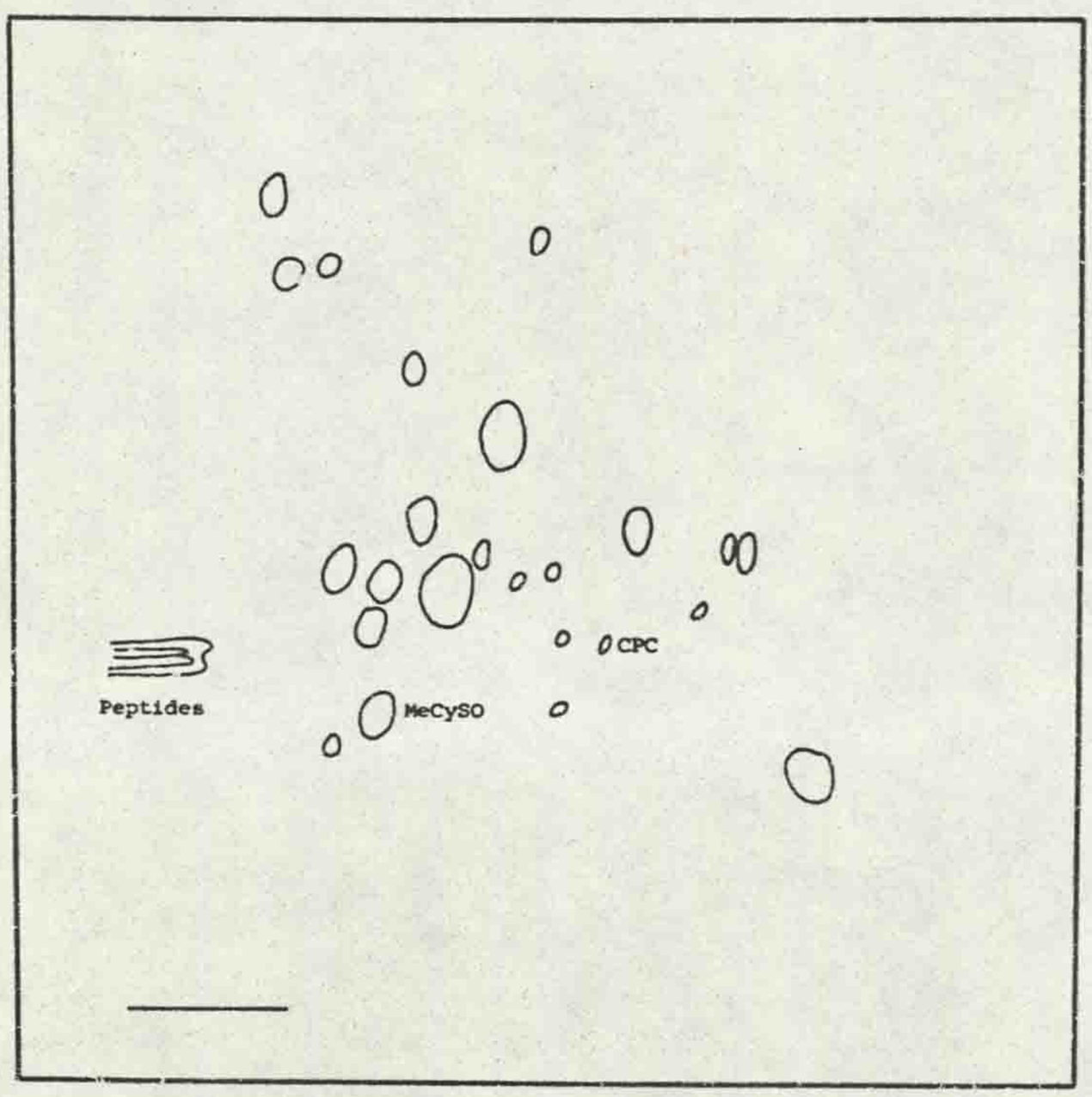
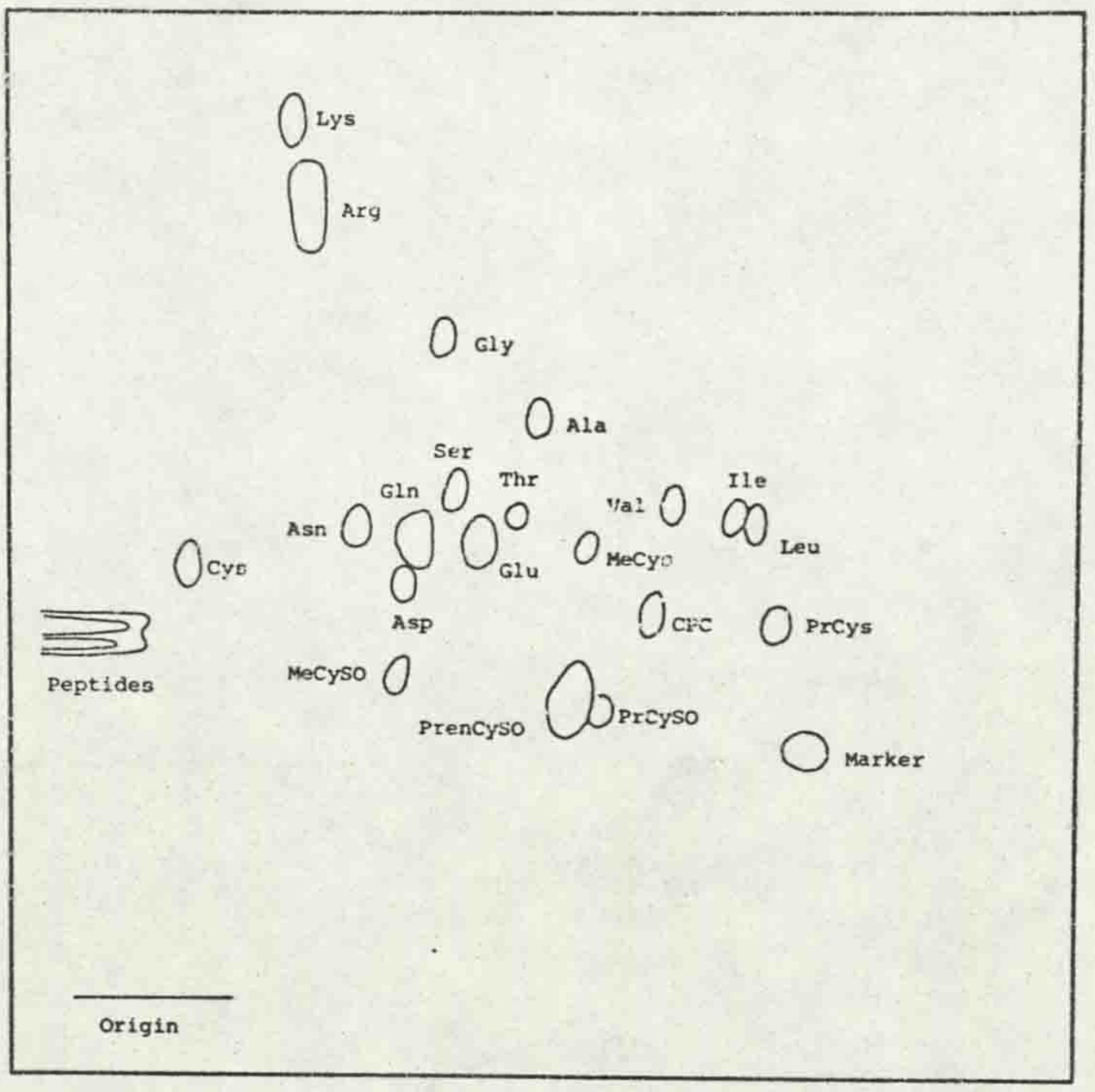


Fig. 3.3. Map of the distribution of free amino acids and flavour compounds on thin layers.

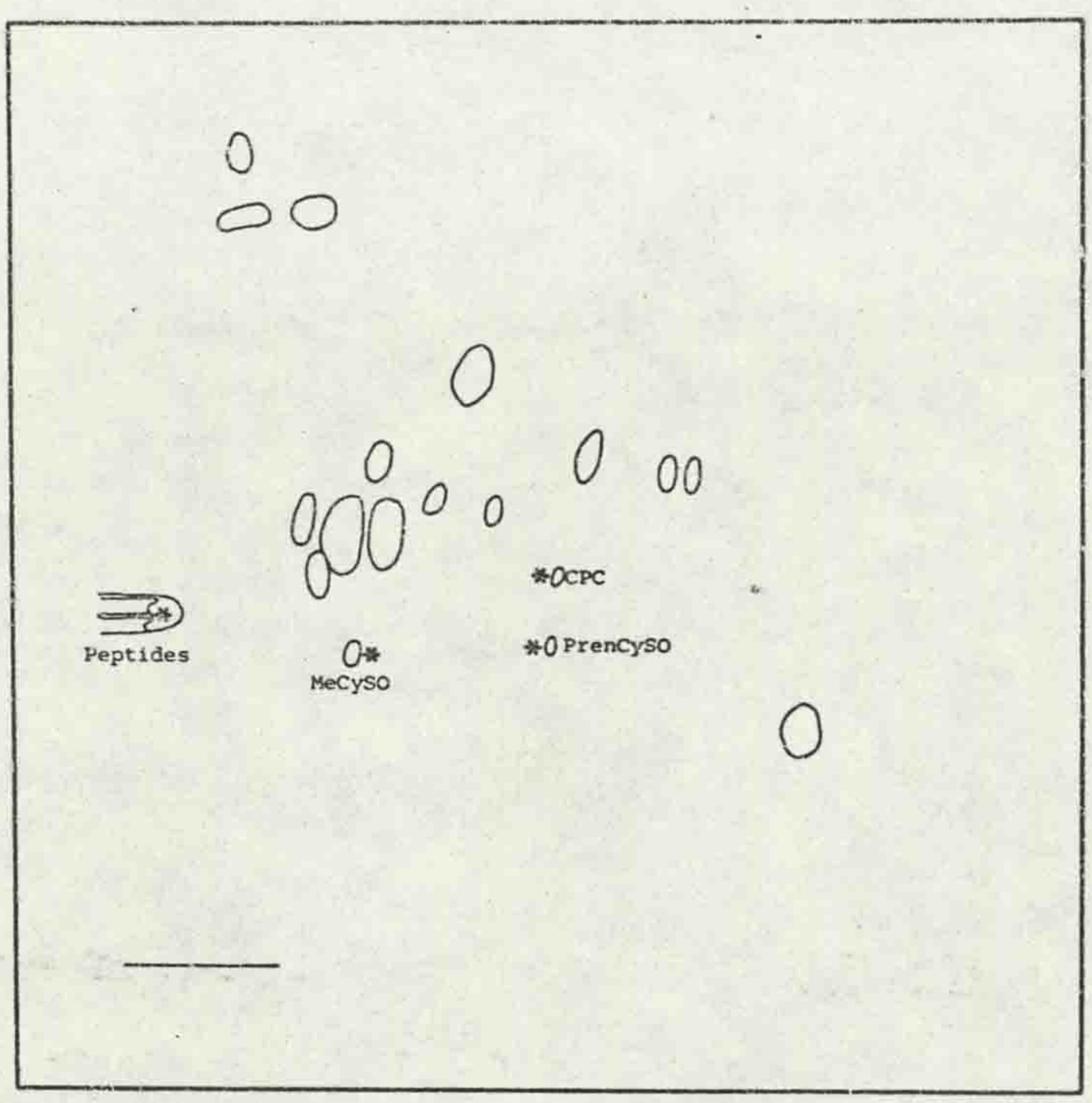

Lys
Arg
Gly
Ala
Ser
Gln
Thr
Asn
Val
Ile
Leu
Cys
MeCys
Glu
Asp
CPC
PrCys
Peptides
MeCySO
PrCySO
PrenCySO
Marker
Origin

^{14}C - cysteine was metabolised more rapidly by onion shoot tips into a wider range of compounds (Fig. 3.4b). The pattern of labelling was similar for two and four day samples although some compounds such as asparagine, glutamine and aspartic acid were more heavily labelled after four days. Me Cy SO and Pren Cy SO were heavily labelled in both cases, most of the label being in the latter compound. Other amino acids labelled were alanine, valine, isoleucine, leucine, serine, glutamic acid, CPC and peptides. These observations indicate the incorporation of cysteine into Pren Cy SO and Me Cy SO as the main flavour compounds of the onion, with more label accumulating in Pren Cy SO. The pattern of amino acid labelling in the shoot tip was basically the same as that of callus tissue but in the former case more free aminoacids and ninhydrin negative compounds were labelled. In both callus and onion tissues, no free cysteine was observed, indicating that it is rapidly incorporated and metabolised into two or three main end products, namely Me Cy SO, Pren Cy SO and peptides.

When ^{14}C - serine was fed to callus, several amino acids were labelled after only three days incubation (Fig. 3.5a). These included arginine, glycine, alanine, leucine, isoleucine, asparagine, serine, glutamine, glutamic acid. Me Cys, Pren Cy SO, Me Cy SO and peptides, with only the last two besides serine being heavily labelled. After seven and fourteen days, fewer free amino acids were labelled but otherwise the pattern was the same with heavy labelling of Me Cy SO and peptides and some labelling of CPC and Pren Cy SO. As before, these results indicate operation

Fig. 3.4 Thin layer chromatogram of extracts from tissue supplied with ^{14}C-cysteine, a) onion callus (7 days), b) onion shoot tip (4 days).
* ^{14}C label.

a


Peptides
MeCySO
CPC
PrenCySO

b

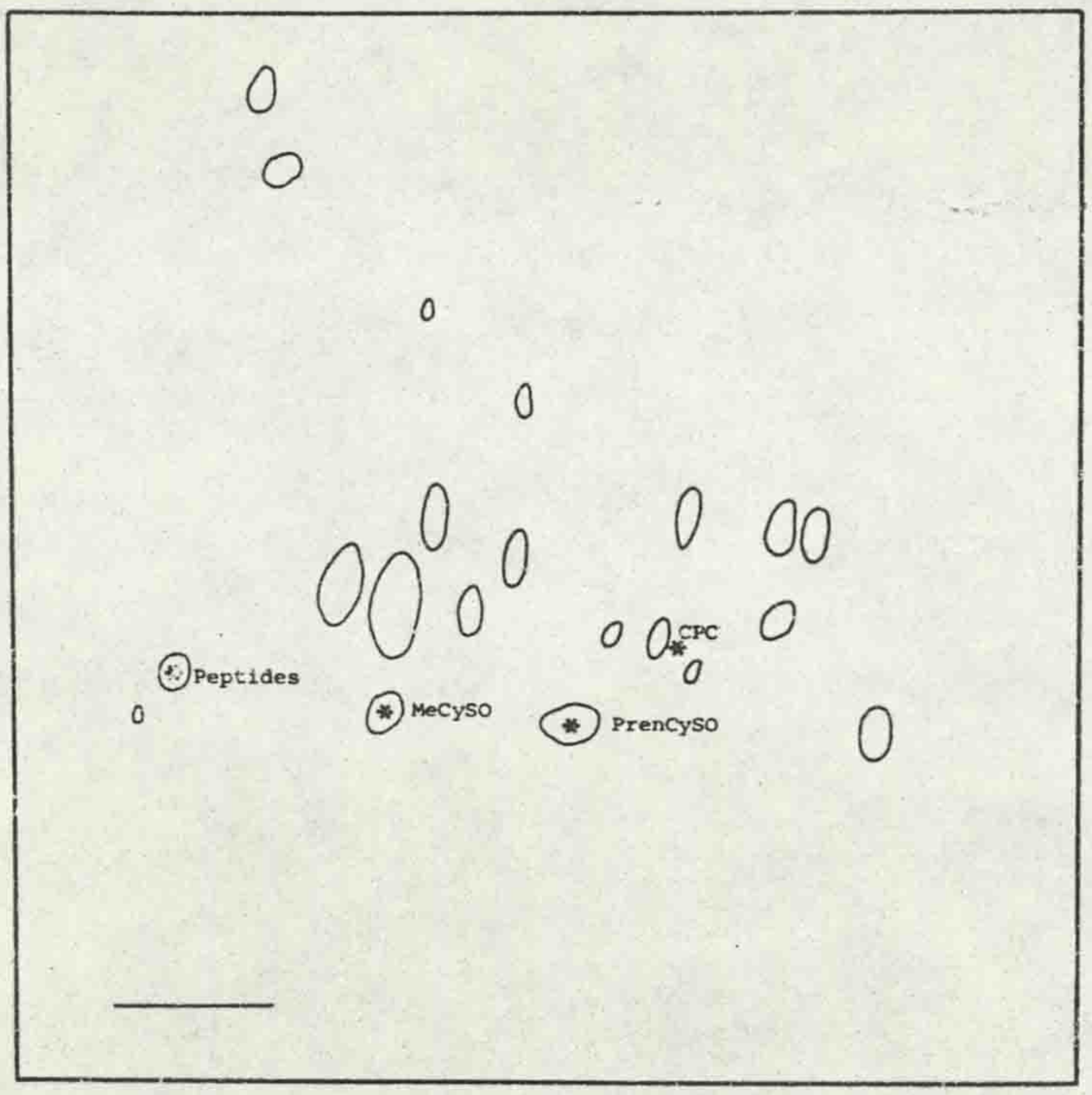
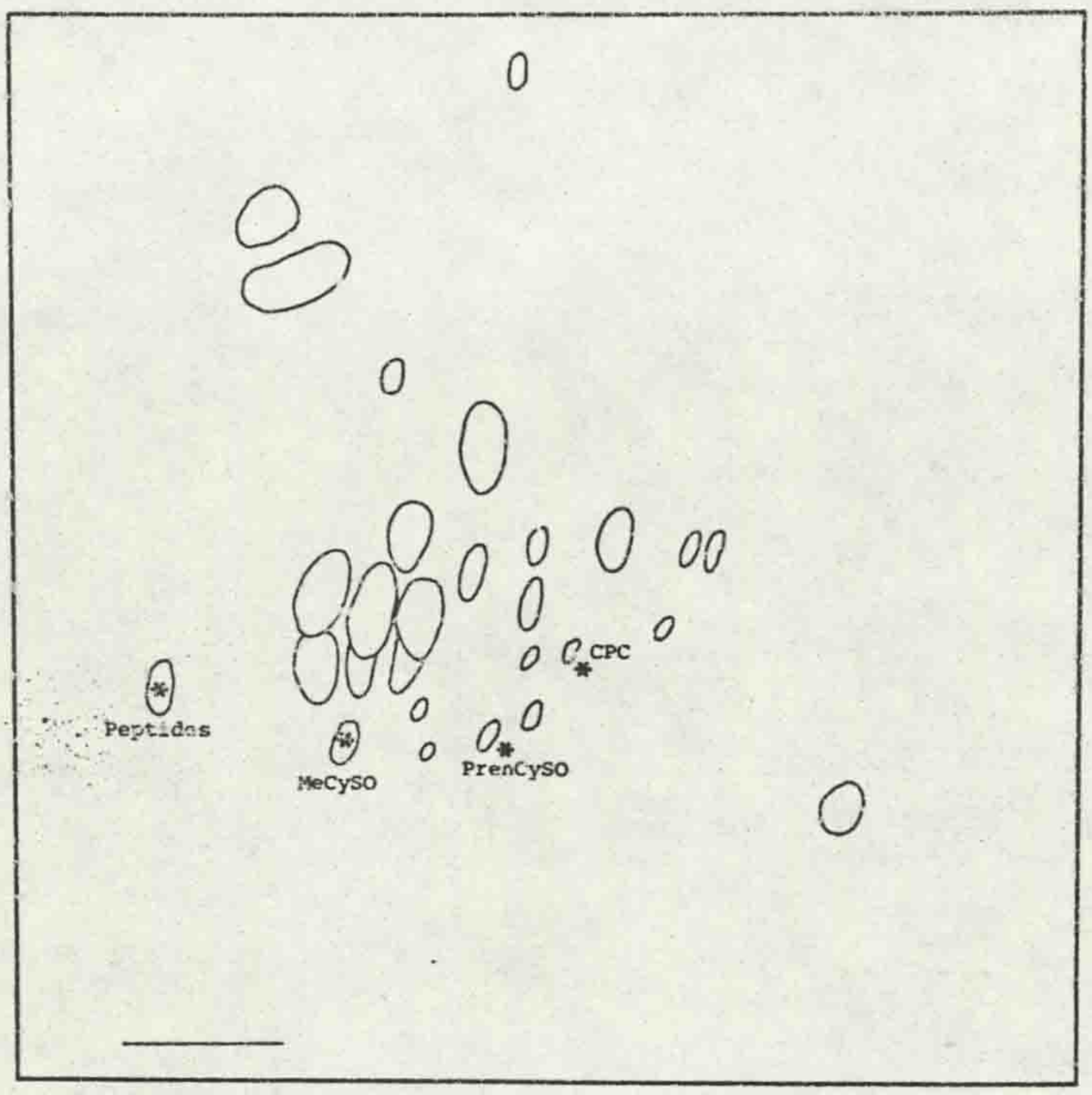
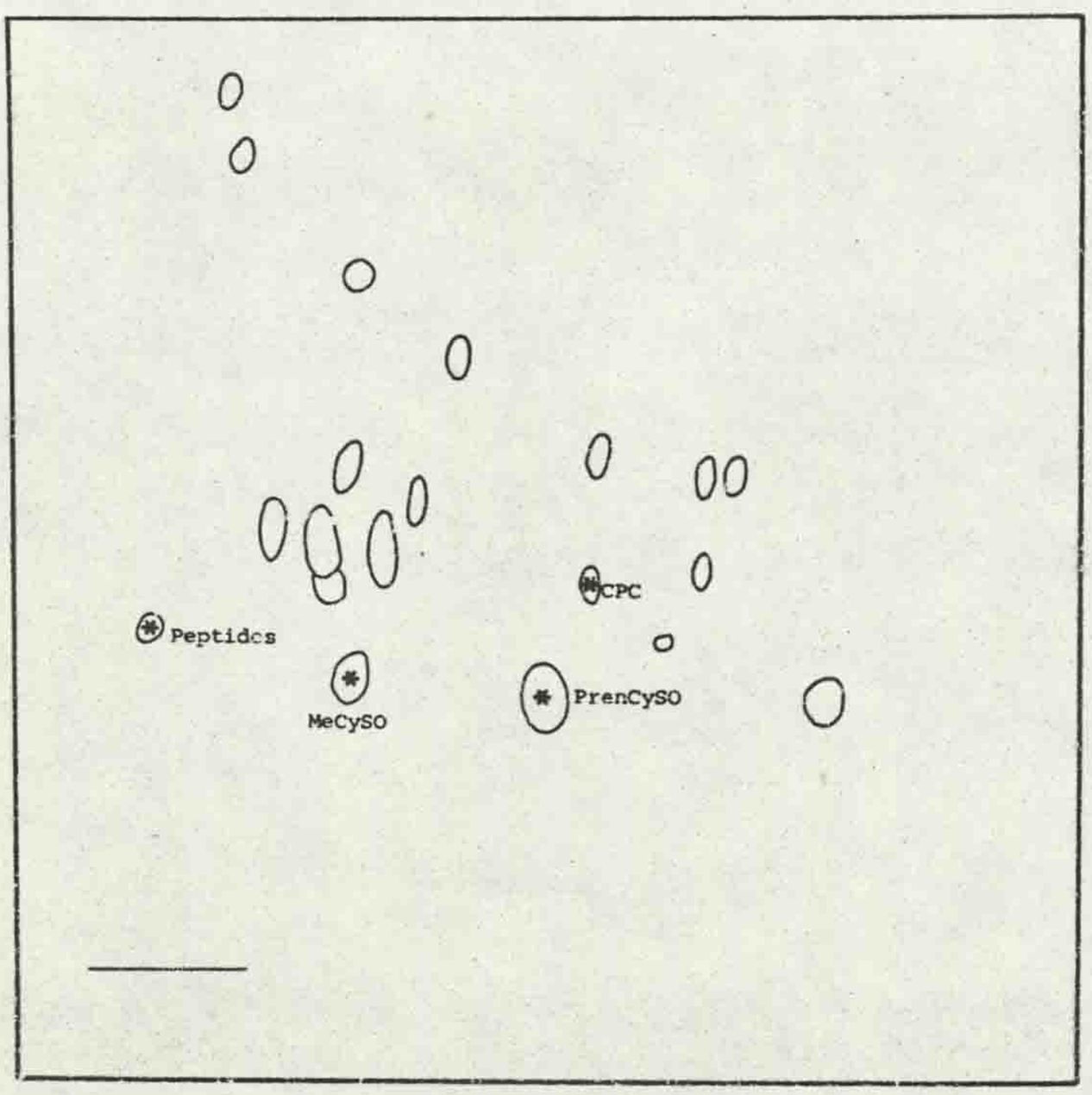

Peptides
MeCySO
CPC
PrenCySO

Fig. 3.5 Thin layer chromatogram of extracts from tissue supplied with ^{14}C-serine, a) onion callus (3 days), b) onion shoot tip (2 days).
* ^{14}C label

a


CPC
Peptides
MeCySO
PrenCySO

b


CPC
Peptides
MeCySO
PrenCySO

of the biosynthetic pathway in callus resulting in the formation of Pren Cy SO. The labelling of CPC and Pren Cy SO support this hypothesis. The heavy labelling of peptides in both cases suggests that cysteine and serine are metabolised to peptides which may act as storage compounds. Heavy labelling of Me Cy SO was expected since this seems to be the main flavour compound of callus tissue. It was always present in callus in large amounts as were the peptides mentioned above.

Since serine and cysteine are virtually interchangeable in terms of amino acid metabolism, similar patterns of incorporation were expected. The main difference was that serine was incorporated into several ninhydrin negative compounds, which, in the confines of this experiment, were not identified.

The feeding of labelled serine to shoot tips (Fig. 3.5b) resulted in a similar distribution of label as in the case of cysteine, with a wider range of free amino acids being labelled. These included arginine, glycine, threonine and Me Cys as well as those listed for cysteine-fed material. As in the case of cysteine, Me Cy SO, peptides and Pren Cy SO were all heavily labelled. The same conclusions can therefore be drawn from both of these experiments. In general, incorporation of cysteine and serine occurred into a wider range of free amino acids when they were fed to onion shoot tips than when fed to callus.

^{14}C - valine was not metabolised by callus tissue during the course of the experiment. The only labelled spots observed were those of valine and organic acids (Fig. 3.6a). Preliminary

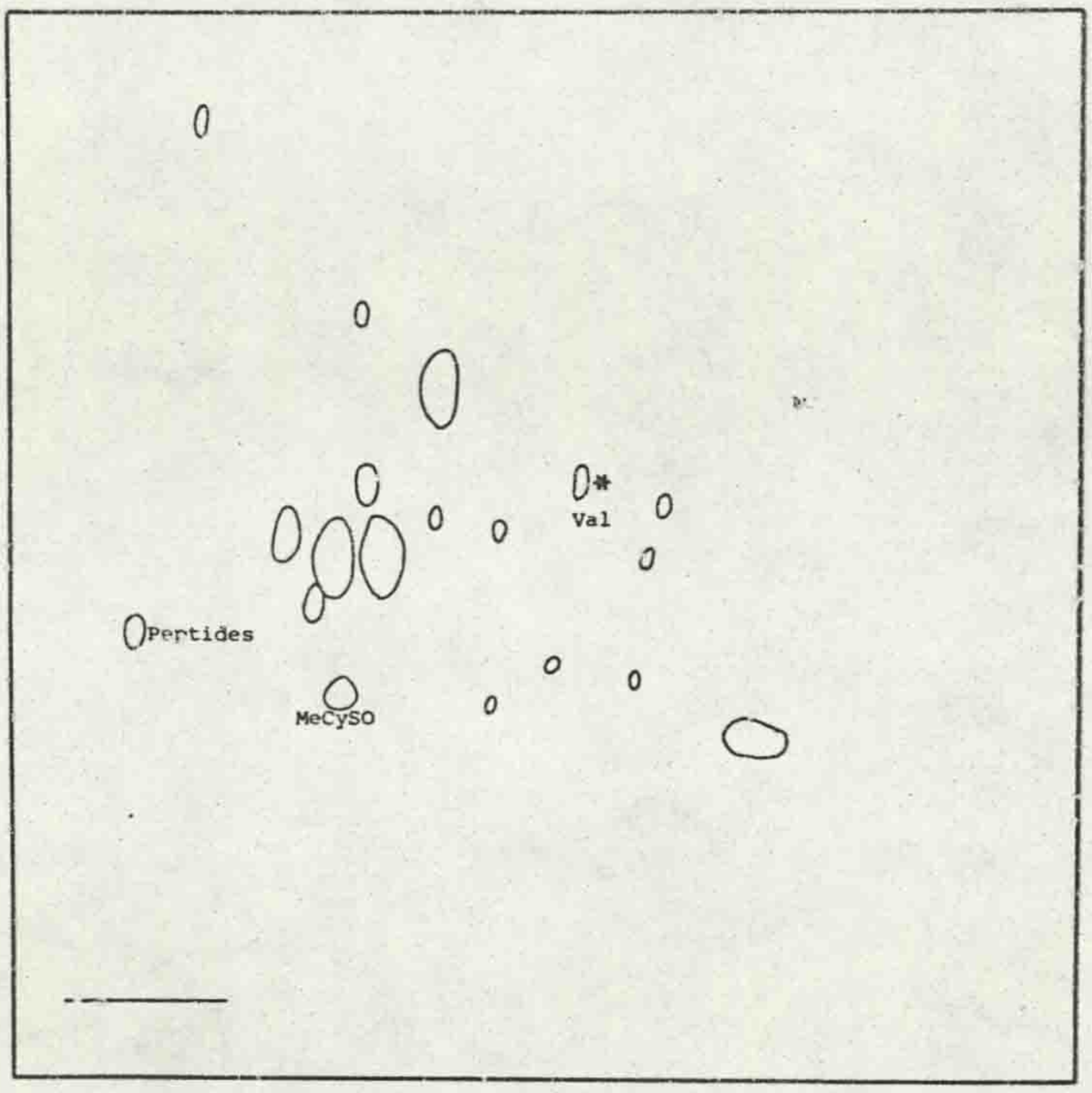
Fig. 3.6 Thin layer chromatogram of extracts from tissue supplied with ^{14}C-valine,

a) onion callus, (3 days),

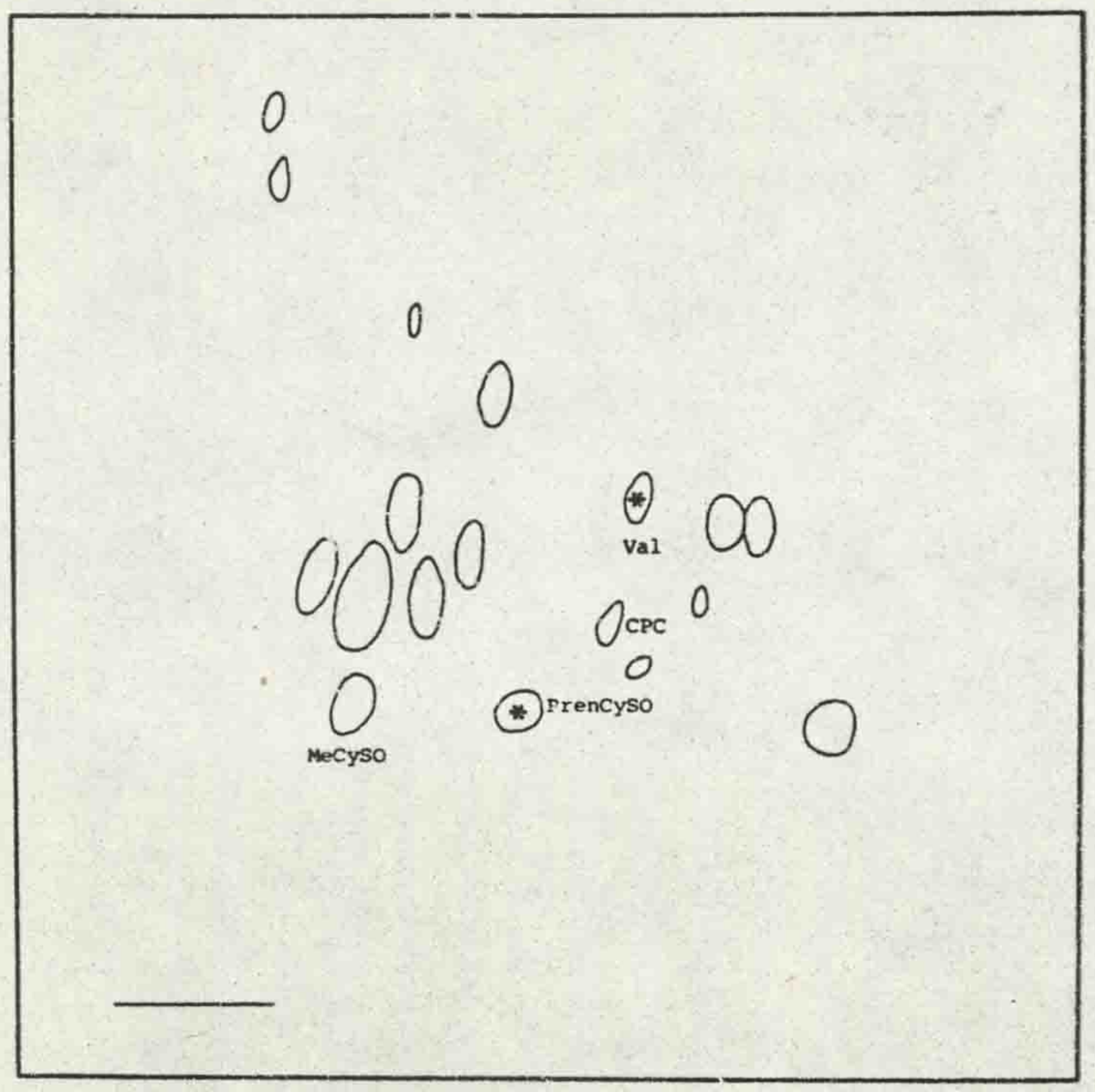
b) onion shoot tip (4 days)

* ^{14}C label

a



b



experiments where sampling was done at intervals of up to three days showed slight labelling of serine, glutamine, CPC and Me Cys but after three days no labelling of any compound except organic acids was recorded.

Similar results were obtained when ^{14}C - valine was fed to onion shoot tips. After two days only serine was labelled whilst after four days several free amino acids were labelled (Fig. 3.6b). These included glycine, alanine, asparagine, serine, threonine, glutamic acid and aspartic acid. Heavier labelling was observed in Pren Cy SO. These results show that ^{14}C - valine was slowly metabolised by actively growing onion tissue to produce the main flavour compound, Pren Cy SO. Uptake and metabolism of valine was not as rapid as that of serine or cysteine, as seen by the large number of radioactive compounds detected in the latter two cases.

The feeding of labelled cysteine and valine to onion bulb slices was unsatisfactory due to the very slow metabolism of this tissue. Valine was not metabolised at all during the three days of the experiment, except to a small amount of organic acids. Cysteine was metabolised to a greater extent but only slight labelling of Pren Cy SO and glutamine were observed. Better results may have been obtained using slices of sprouting bulb tissue, or by allowing a longer incubation time and feeding a higher level of label. However, a longer experimental time would have increased the risk of contamination. The centre part of the bulb was used since this is virtually free of contamination but the tissue was not

surface sterilised in case of resulting tissue damage.

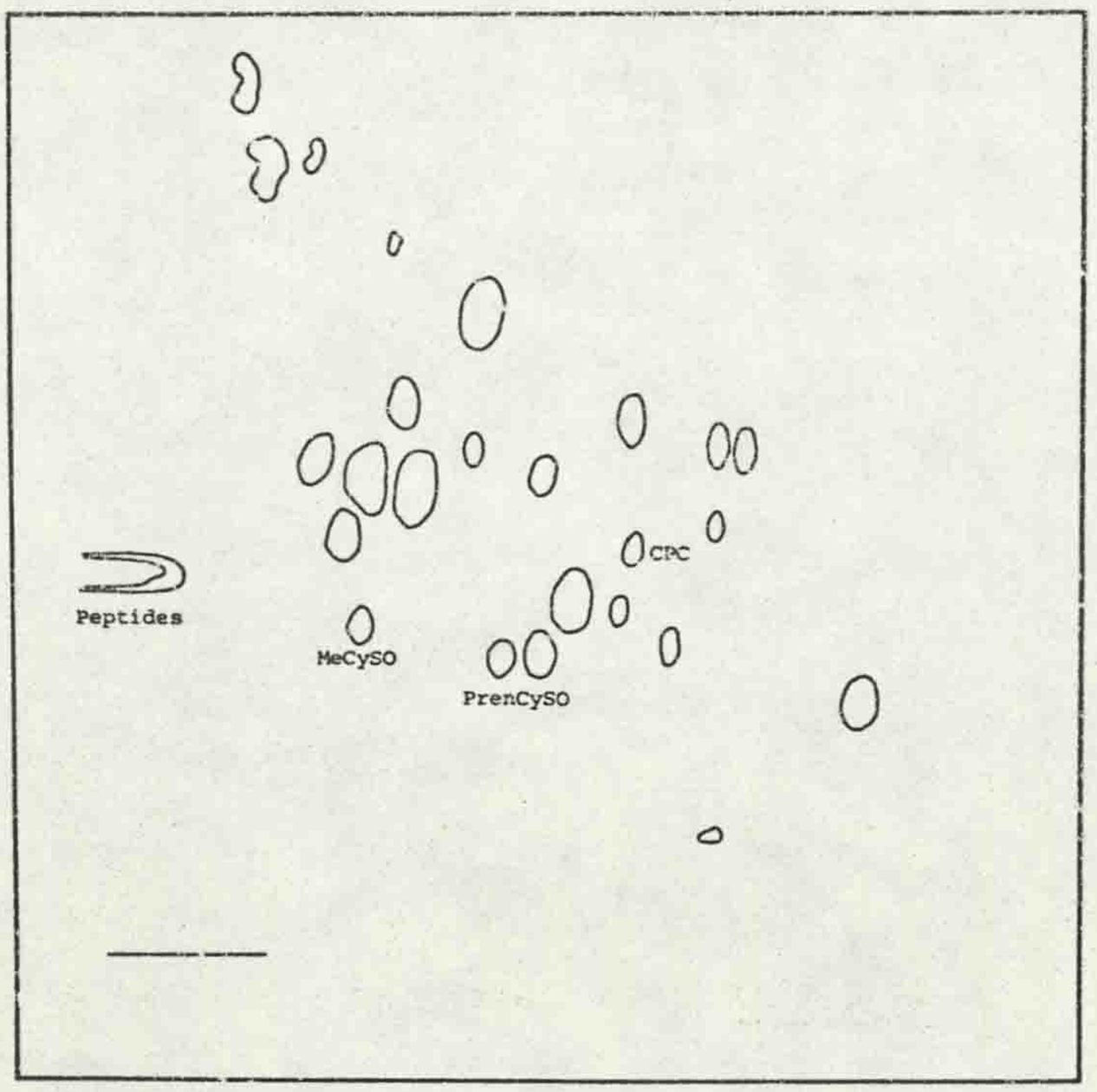
Some of the radioactive compounds which were apparently ninhydrin negative may have been present in trace amounts only. This could be confirmed by feeding a higher level of radioactivity and using longer sampling times. Thin layers are very susceptible to overloading and so it was not possible to apply more extract to the plate since overloaded spots tend to 'tail' and obscure other compounds. The use of a more sensitive reagent such as fluorescamine would also facilitate identification of those compounds present in trace amounts which were not detectable with ninhydrin.

3.3iii Feeding of Cysteine Derivatives.

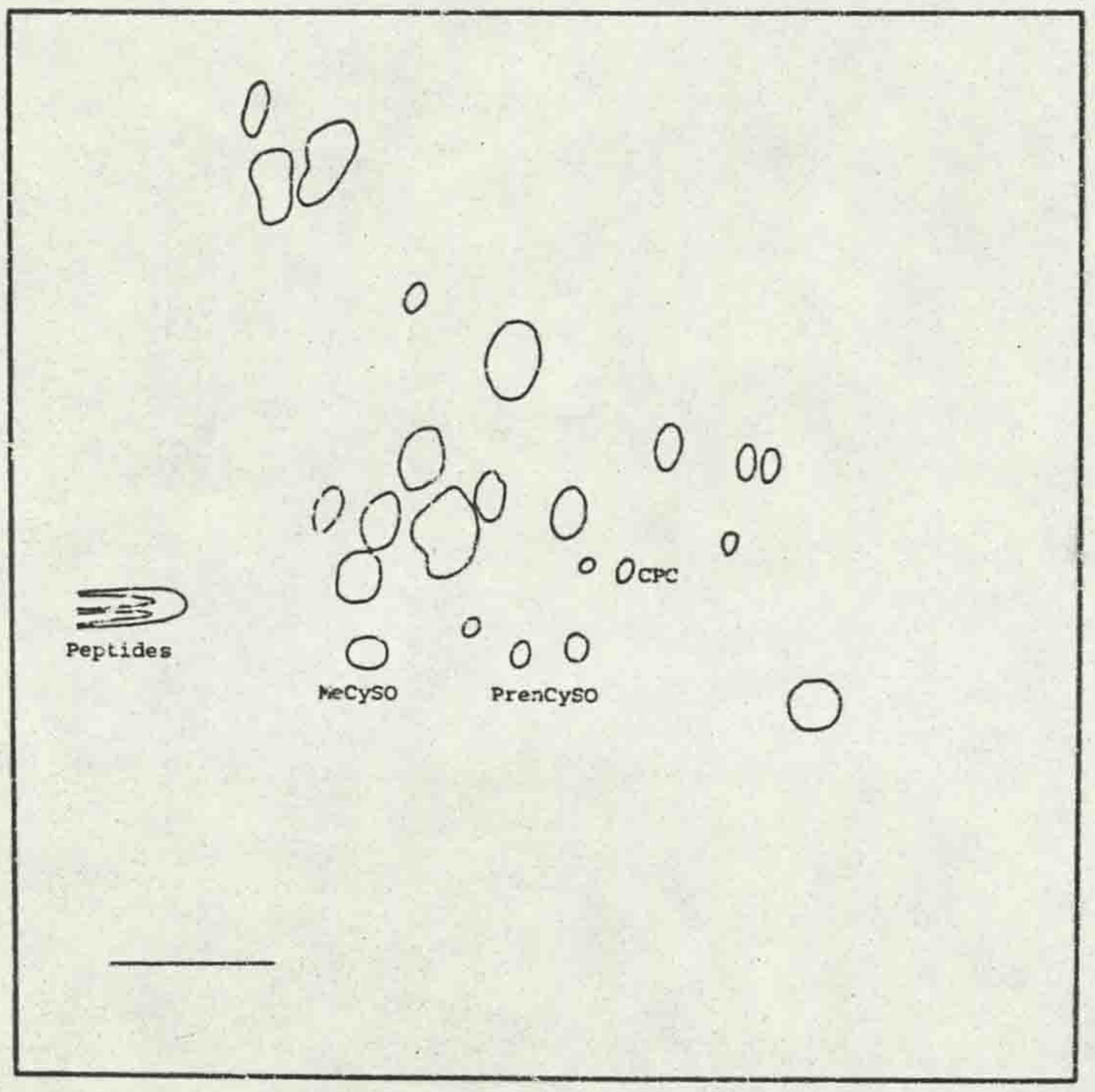
Fig. 3.7a shows the distribution of amino acids obtained when an extract of CPC fed callus was analysed by TLC - electrophoresis. This shows clearly the presence of CPC and Pren Cy SO in the extract. A similar pattern was obtained when Pren Cys was fed to callus (Fig. 3.7b). These results therefore support those of the radioisotope feeding experiments where it was demonstrated that the biosynthetic pathway was operating in callus tissue. In each case, two ninhydrin positive spots were present on the TLC plate in the expected position of Pren Cy SO. These two spots corresponded to cis-(+)-Pren Cy SO and trans-(+)-Pren Cy SO. These results are in agreement with the results of experiments done by Granroth (1970), where synthetic ^{35}S-CPC was fed to chive leaf tissue and label was found incorporated into the two Pren Cy SO isomers. This was because synthetic

Fig. 3.7 Thin layer chromatogram of extracts from onion callus tissue supplied with a) CPC (7 days), b) Pren Cys (7 days).

a


Peptides
MeCySO
PrenCySO
CPC

b


Peptides
MeCySO
PrenCySO
CPC

CPC consists of a mixture of two diastereoisomers which are metabolised in different ways to produce both the cis isomer and the trans isomer of Pren Cy SO. Carson et al. (1966) showed that the naturally occurring isomer of Pren Cy SO has the trans configuration.

The synthesis of Pren Cys resulted in a mixture of the two diastereoisomers in which trans Pren Cys was present in small amounts only (Carson and Wong 1963). No method was available for the synthesis of a high yield of the trans isomer and so the mixture was used in the feeding experiments. This explains the appearance of two spots on the TLC plate in the position of Pren Cy SO.

Pren Cy SO was prepared from the mixture of the two Pren Cys isomers which presumably resulted in the production of the two isomers of Pren Cy SO. After three days in the presence of Pren Cy SO, an extract of the callus produced only one spot in the expected position on the TLC plate (Fig.3.8) whilst after seven days, two spots were detected. This may reflect a difference in the rates of uptake by the callus tissue of the two isomers. Exogenously supplied Pren Cy SO appeared to be taken up and accumulated by the callus. No onion smell was detected in the culture flasks indicating that the Pren Cy SO mixture was not degraded by the callus.

Tables 3.2 and 3.3 show the levels of amino acids and flavour compounds recorded in callus tissues after feeding CPC (Table 3.2), Pren Cys and Pren Cy SO (Table 3.3). In CPC

Fig. 3.8 Thin layer chromatogram of extracts from onion callus tissue supplied with Pren Cy SO (3 days).

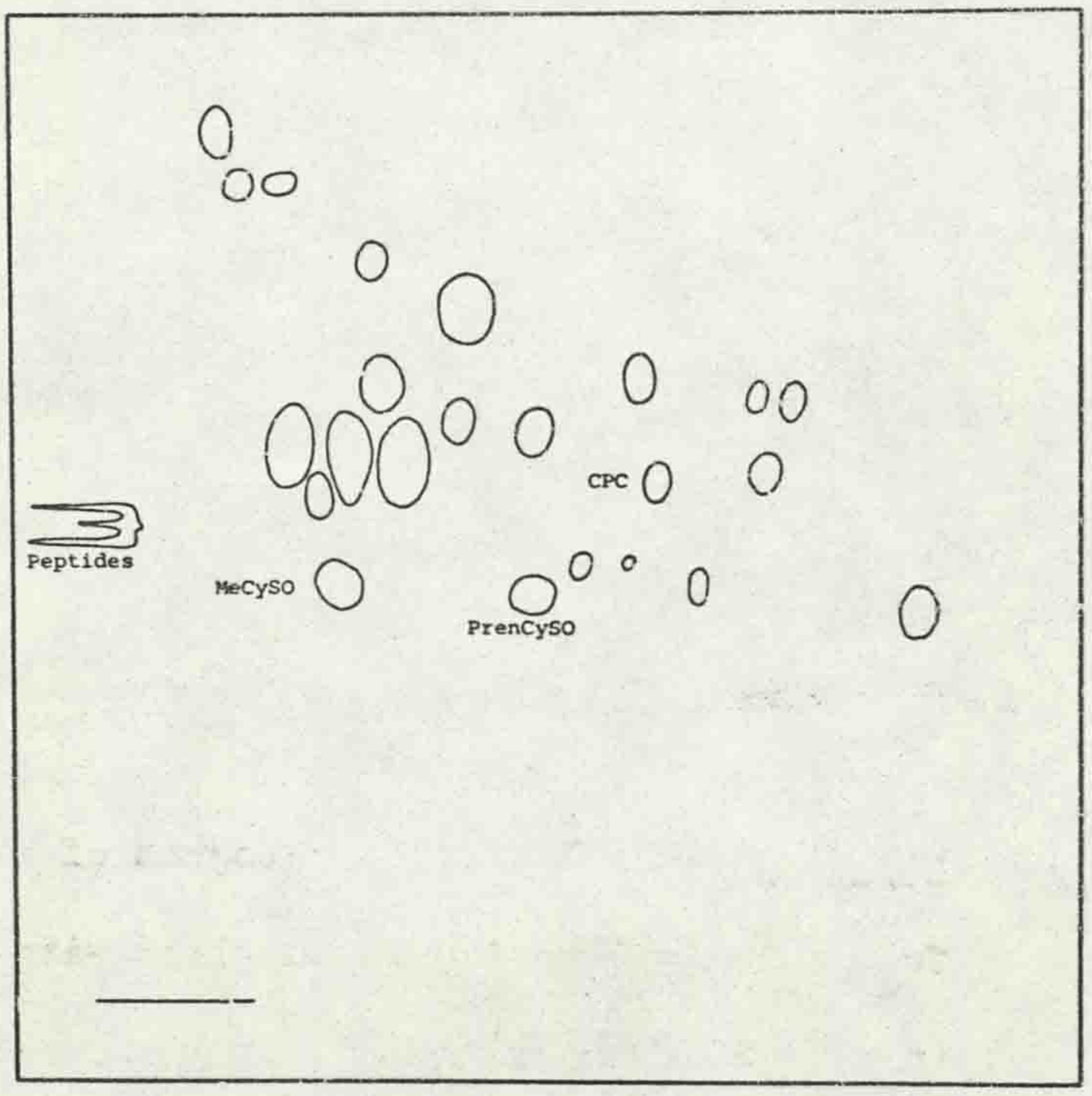

Peptides
MeCySO
CPC
PrenCySO

Table 3.2 Levels of amino acids and flavour compounds in samples of callus fed with synthetic CPC.

Amino acid	μ mole g^{-1} fresh weight		
	Callus sample		
	5 days	7 days	14 days
Lysine	0.12	0.14	0.07
Histidine	0.03	0.05	0.04
Me Cy SO	24.80	21.50	12.00
Pren Cy SO	Trace	Trace	86.56
Arginine		0.41	0.43
Asparagine	1.20	1.40	
Threonine	1.80	3.58	
Serine	1.18	0.88	
Glutamic acid	6.74	5.82	2.11
CPC	1.49	3.71	Trace
Pren Cys		12.82	Trace
Glycine	0.14	0.19	0.16
Alanine	2.22	2.79	3.23
Valine	0.51	0.56	0.57
Isoleucine	0.06	0.08	0.16
Leucine	0.18	0.22	0.15
Tyrosine	0.05		0.06
Phenylalanine			0.04

Table 3.3 Levels of amino acids and flavour compounds in samples of callus fed with synthetic Pren Cys or Pren Cy SO.

Amino acid	μ mole g^{-1} fresh weight				
	Callus sample				
	Pren Cys			Pren Cys SO	
	3 days	7 days	14 days	3 days	7 days
Lysine	0.11	0.18	0.21		
Me Cy SO		1.40			
Pren Cy SO	2.20	15.23	19.30	0.55	7.29
Arginine		0.52	1.24		0.11
Asparagine		0.73	1.07	0.07	0.74
Threonine		2.80	2.17	0.05	0.69
Serine	0.28	2.07	2.10	0.13	0.71
Glutamic acid	0.59	7.95	9.35		
CPC		10.61	18.83	3.59	54.14
Pren Cys		15.07	34.43		
Glycine	0.09	0.39	0.31		0.14
Alanine	0.68	11.69	10.26		1.23
Valine		0.87	0.86		
Methionine		0.09	0.10		
Isoleucine		0.11	0.13		
Leucine		0.30	0.36		
Tyrosine			0.08		

fed tissue, it was shown that Pren Cy SO was present in trace amounts at days 5 and 7 but this level increased dramatically 14 days after feeding. The level of CPC detected after 7 days was double that of the 5 day sample and Pren Cys was detected after 7 days, but both of these compounds were present in trace amounts only after 2 weeks. These results indicate that CPC was taken up by the callus and converted to Pren Cy SO since the latter was present in significant amounts 2 weeks after feeding CPC. The level of CPC in the callus samples increased from days 5 to 7 as uptake of CPC continued and as this was metabolised, Pren Cys was also detected at day 7. However, both of these compounds had disappeared from callus by day 14 as they were converted to Pren Cy SO.

Similar results were recorded for the experiment in which Pren Cys was fed to callus. Pren Cys was found to be present in increasing amounts in the 7 and 14 day samples whilst Pren Cy SO was found in all samples at levels which increased with time. For example, there was a 7 fold increase in Pren Cys SO between days 3 and 7 and a 9 fold increase between days 3 and 14. When Pren Cy SO was fed to callus, this compound was detected in the 3 and 7 day samples during which time the level increased 13 fold. However, in both of these feeding experiments the level of CPC in the callus also increased with time. For example, a 15 fold increase in the amount of CPC present between days 3 and 7 was observed when Pren Cy SO was fed.

It was calculated that the level of CPC normally found in

callus tissue was approximately 1.5 μ moles g^{-1} fresh weight whilst in these experiments where Pren Cys and Pren Cy SO were fed, levels up to 54 μ moles g^{-1} fresh weight were recorded. Thus, it was apparent that the callus was synthesising CPC since no CPC had been exogenously supplied and the levels were up to 36 times greater than those normally found in the callus. Support for this hypothesis was obtained from the discovery that the levels of Pren Cys recorded in the tissue which had been fed this compound were in excess of the amount of Pren Cys actually supplied. Similarly, the level of Pren Cy SO recorded 7 days after the sulphoxide was fed, was in excess of the amount of this compound supplied. Therefore, feeding of Pren Cy SO or its immediate precursors to callus was apparently inducing synthesis of this sulphoxide.

Another interesting point to note was that in the callus fed with CPC, the level of Me Cy SO in the tissue steadily declined over the three sampling times. Only trace levels of Me Cy SO were detected in most of the samples which had been supplied with either Pren Cys or its sulphoxide. The free amino acids in these samples appeared to remain relatively constant throughout the experimental period although glutamic acid was seen to decline markedly over the 14 days of CPC feeding but increase steadily over a similar period of Pren Cys application.

3.4 Discussion

The results of the high level feeding experiments suggested that only the biosynthetic pathway from CPC to Pren Cy SO was operating in callus because the flavour of onion was obvious when CPC fed tissue was crushed, whilst no onion odour was produced when intermediates from the earlier part of the pathway were supplied. However, results of the radioisotope feeding experiments showed that the earlier part of the pathway was operating in callus, but more slowly than in the onion tissue. The pathway was not blocked as originally believed since ^{14}C - cysteine was converted to CPC. Labelling of CPC and Pren Cy SO showed that synthesis of Pren Cy SO can occur in callus and that the Pren Cy SO so formed may be bound immediately to form peptides, possibly of the glutathione type, since these were always labelled. Labelling of Me Cy SO indicated that this metabolic pathway was also active in callus tissue.

Me Cy SO is the main flavour precursor in callus tissue in the same way as All Cy SO predominates in garlic and Pren Cy SO in the onion plant. Thus, these experiments showed that the two main pathways were operating in callus, the synthesis of Me Cy SO and of Pren Cy SO, with the former normally being more important in unorganized callus tissue. It is probable that both products are also bound as peptides. Feeding of radioactively labelled Me Cys, Me Cy SO, Pren Cys and Pren Cy SO would confirm this point.

When CPC, Pren Cys and Pren Cy SO were fed to callus, the TLC patterns showed that CPC and Pren Cys were converted to Pren Cy SO and that Pren Cy SO was taken into the tissue. Feeding of ^{14}C Pren Cy SO would confirm whether this compound was degraded, metabolised to CPC or bound in the form of peptides as a storage product.

Amino acid analysis confirmed that these same three compounds were taken up by the tissue and metabolised. However, it was also shown that exogenous application of Pren Cy SO or its direct precursors seemed to stimulate synthesis of the end product of the biosynthetic chain. This appeared to occur rapidly when the end product, Pren Cy SO was fed, but more slowly with CPC application. For example, the feeding of Pren Cy SO led to the synthesis of large amounts of Pren Cy SO in only 7 days. Similarly, exogenous Pren Cys was used up in 3 days so that no Pren Cys could be detected at this time although some sulphoxide was found in this sample. However, after 7 and 14 days, large amounts of Pren Cys were present which could only be accounted for by'de novo'synthesis of this compound. The results of the CPC feeding experiment also follow this pattern with a longer time lag than those observed in the other two feeding experiments. Thus, it is now believed that exogenous CPC is taken up by the tissue and converted to Pren Cy SO which then stimulates the callus cells to produce more of this compound by'de novo' synthesis. However, extended CPC feeding experiments need to be carried out to finally confirm this suggestion, since it appears

that in the two weeks of the experiment, only the early part of the pattern is obvious. Prolonged incubation of CPC - fed tissue would be expected to result in significantly increased levels of Pren Cy SO, in excess of the initial amount of CPC supplied, as was found in the feeding of Pren Cy SO.

Therefore, a similar pattern was observed in each of the three feeding experiments, with a lag phase which appeared to increase in length according to the position of the exogenous compound in the biosynthetic chain, in relation to the final product. Thus, a rapid response was produced when the end product itself was supplied.

It is suggested that the presence of Pren Cy SO in callus tissues induces an increased synthesis or activation of the enzymes necessary for rapid synthesis of this compound. This explains why the lag phase before the increased synthesis is achieved is longer when CPC is supplied than with direct Pren Cy SO feeding. It should be possible to test this hypothesis by extraction of the natural precursors from onion tissues for use in feeding experiments instead of using synthetic compounds as described in this investigation. By feeding various known amounts of each precursor to callus as described here and assaying the tissue after several time intervals, it should be possible to provide confirmation for the system suggested from the results of these earlier experiments. Similarly, increased synthesis may be demonstrated by the use of gel electrophoresis techniques. If the

hypothesis is correct,more intensely staining bands of protein will appear in precursor-treated callus extracts due to the synthesis of more enzyme. However, if no change occurs in such an experiment it would imply that the effect of exogenous precursors on callus tissue is simply activation of existing enzymes rather than increased synthesis of these enzymes.

Activation of existing enzymes is a likely possibility in view of the results of the radioisotope feeding experiments which demonstrated that incorporation of low levels of precursors into Pren Cy SO was occurring. However, both synthesis and activation of enzymes may occur in the treated callus so that conversion of precursors to Pren Cy SO occurs as a result of the required enzymes being present in the callus. Presence of increased levels of Pren Cy SO so formed then stimulates further synthesis of these same enzymes.

It is thought that the decline in the level of Me Cy SO in CPC treated tissue suggests that the callus was switching over to a different biosynthetic pathway so that Pren Cy SO would replace Me Cy SO as the predominant flavour precursor in callus tissue. It would be interesting to see if the decline in Me Cy SO synthesis continued with longer term incubation of CPC fed callus and also if the effect was permanent. Similarly, it would be interesting to see how long the callus continued to synthesise Pren Cy SO after the initial stimulation was produced by providing exogenous precursor.

CHAPTER 4

FLAVOUR PRODUCTION IN DIFFERENTIATING CALLUS CULTURES

4.1 Introduction

The bulk of evidence from tissue culture studies points to the potential toripotency of plant cells in terms of morphogenesis. Thus, it is likely that these cells are also biochemically totipotent so that every cell will contain the genetic information necessary to reproduce the biochemistry of the whole plant (Krikorian and Steward 1969). Some plant cells in culture have been reported to produce significant amounts of the secondary products characteristic of the parent plant. Examples include the production of alkaloids by Vinca rosea callus (Harris et al. 1964) and Alstonia constricta callus (Carew 1965) and anthraquinone by cell suspensions of Morinda citrifolia (Zenk et al. 1975). There are also reports of synthesis of secondary metabolites which were not detectable in the whole plant, e.g. Butcher and Connolly (1971) detected three novel sesquiterpenes in callus cultures of Andrographis paniculata whilst the typical diterpenes of the intact plant were absent. However, in the majority of cases, cultured tissues synthesise the same secondary metabolites as those of the whole plant, but only at low levels, with a maximum of 10% of the level found in the plant (Puhan and Martin 1971). In fact, there is a striking uniformity in the synthetic capacities of a wide variety of cultured tissues. In general, the metabolic activities of cultured cells appears to be directed towards primary metabolism and the synthesis of compounds necessary for cell division and growth (Turner 1971). However, it is still

unknown if the low level of secondary compound production is a result of synthesis occurring in a few cells only, or if it reflects a slow rate of synthesis in every cell of a population (Street 1973).

It is a common phenomenon in plant tissue culture that secondary products only appear in significant amounts when the tissue becomes differentiated and organized to form roots, shoots and plantlets. In other words, there is a restoration of secondary metabolism in plant cultures in which organization has occurred. Examples include the synthesis of alkaloids in roots of Peganum harmala callus (Nettleship and Slaytor 1974) and regenerated plantlets of Papaveraceae species (Ikuta et al. 1974) and Datura innoxia (Hiraoka and Tabata 1974) and of cardenolides in regenerated shoots of Digitalis purpurea (Hirotani and Furuya 1977). However, it is still a matter for conjecture whether morphogenesis prescribes the pattern of secondary metabolism or if the latter is a determinant in the process of morphogenesis and development.

Nevertheless, there is some relationship between the presence of structurally modified and organized tissues in cultured callus and the synthesis of secondary metabolites by those cultures. The present study was undertaken to find out if a similar relationship existed in the onion cultures.

Several investigations have been carried out on onion callus tissue and have included work on callus derived from

bulb scales (Fridborg 1971, 1974, Nandi et al. 1977), root explants (Davey et al. 1974), basal disc explants (Freeman et al. 1974) and root tissue (Selby and Collin 1976). Dunstan (1977) obtained callus cultures from the bulb, onion sets, seedling roots and flower buds. Most of these studies were concerned with factors affecting morphogenesis. In general, the presence of 2,4-D in the culture medium promoted unorganized callus growth (Nandi et al. 1977) whilst transfer of the callus to medium in which 2,4-D was replaced by NAA stimulated root formation (Fridborg 1971, Freeman et al. 1974). Shoot formation was reported by Mackenzie and Davey (Unpublished results), Fridborg (1971, 1974) and Dunstan (1977). In each case shoot formation was difficult to control by chemical means and was reported to occur only in young callus cultures. Fridborg (1971) reported that leafy buds occurred only sporadically on tissue which had been in culture for one year although root forming ability was retained.

Fridborg (1971) also noted that callus which had differentiated roots produced the characteristic onion odour. Later work by Davey et al. (1974) showed that both undifferentiated and rooting callus failed to produce the flavour components of onion. They also reported a correlation between the level of polyploidy in the cultured tissue, the lack of flavour components and the failure of the callus to regenerate shoots. Callus tissue used in this study had been cultured for more than three years, whilst that used by Fridborg(1971) was less than one year old. Newly initiated callus which had been cultured for only ten months was used by Freeman et al. (1974) in order to test for the presence of flavour components using an extensive series of chemical and sensory tests. They showed that

rooting callus produced flavour intensities approaching that of fresh onion, although some qualitative differences were obvious, particularly in lachrymatory potency. They also demonstrated that the reduced level of flavour compounds in undifferentiated onion callus was due to absence of flavour precursors. Similar findings were reported by Selby and Collin (1976) who showed that the level of flavour precursors in callus was only 2-10% of that found in the onion plant, regardless of the flavour strength of the cultivar used for callus initiation. No information is available regarding the flavour precursors found in regenerated shoots. It is possible that the full flavour complement may be restored when shoots or plantlets are regenerated from callus tissue, including production of the lachrymatory factor which was lacking in rooted callus.

The work reported here was carried out to investigate the flavour complement of roots and shoots regenerated from callus tissue. Differentiated callus tissue was also examined. To compare the system in callus with natural differentiating systems, root and shoot material from a sprouting bulb and young seedlings were tested. Rapidly growing, undifferentiated callus and dormant onion bulb tissues were used as controls. Extracts of sampled material were separated by TLC-electrophoresis to enable identification of the flavour compounds and the results confirmed by amino acid analysis. Where sufficient material was available, the levels of flavour compounds present in the various tissues were measured by estimation of enzymically produced pyruvate. Similarly, a measure of alliinase activity was obtained where possible.

Seedlings were tested at various stages after germination to determine if the characteristic flavour compounds of onion tissues were present at all stages of growth or whether they were synthesised at a particular stage in plant development.

4.2 Materials and Methods

4.2.i Induction of Differentiating Systems

Callus was induced to differentiate into roots and shoots as described in Section 2.2 v. Onion bulbs were induced to produce shoots and roots as described in Section 3.2 ii. For the study of seedling development, onion seeds were soaked in water overnight then sown onto damp cotton wool in covered petri dishes and incubated in a 16 hour light regime at room temperature. Seedlings at various stages after germination were sampled for analysis.

4.2.ii Sampling of Material for Analysis

Extracts of root and shoot material from seedlings, sprouting bulb and callus were prepared as described in Appendix 2i as was material from the centre of a sprouting bulb. Only the distal portions of the roots produced by the callus were sampled, whilst the base of the root where it was attached to the callus mass was discarded. To remove all traces of agar from the roots, they were quickly washed in distilled water then blotted dry between layers of tissue paper before weighing. The callus which had differentiated to form roots was also extracted after all obvious root material had been removed. Only callus shoots of normal appearance were extracted. Differentiating callus which regenerated shoots was not sampled because it was difficult to separate the callus from the differentiated material. Only the green parts of the sprouting onion bulb leaves were used. The bulb roots were

blotted dry before weighing. The non-green portion of the onion seedling between the root and the stem was discarded and only green material extracted for the shoot sample. Roots were separated from all traces of cotton wool before weighing.

Dry onion seeds were coarsely ground in a pepper mill then ground to a fine, oily powder using a pestle and mortar. The powder was mixed with MCW (50 mg ml^{-1}) and left for four hours with stirring. The liquid was decanted, a second volume of MCW added and left with stirring overnight at 25°C. The resulting mixture was centrifuged and the pellet discarded. The combined extracts were then treated with chloroform and water and the procedure continued as described in Appendix 2i, for the preparation of the amino acid extract.

4.2.iii Assay for Enzymically Produced Pyruvate

Pyruvate was measured according to the method of Schwimmer and Guadagni (1962) in which both the endogenous level of pyruvate (Pc) and the level to which the pyruvate increases on disintegration of the tissue (PT) are determined. For measurement of PT, 2.5g tissue were ground up using a pestle and mortar with 2.5 ml 0.1M sodium pyrophosphate buffer, pH 9.0. After standing for 30 minutes at room temperature the homogenate was diluted to 25 ml with distilled water and filtered through Whatman No. 1 filter paper. This procedure was repeated for the measurement of Pc, with the initial homogenate prepared in 2M HCl to prevent enzyme activity.

Estimation of pyruvate

To each 2ml filtrate was added 1ml 0.0125% 2 : 4 dinitro-phenyl-hydrazine in 2M HCl. After incubation for 10 minutes at 37°C, 5ml 0.6M NaOH were added and the optical density at 420nm was recorded on an SP600 spectrophotometer using a reagent blank. A calibration curve (Appendix 4.1b) was constructed using standard solutions of sodium pyruvate up to a maximum of 2 μg ml^{-1}. Results were expressed as μ mol pyruvate ml^{-1} or as specific activity : μ mol mg^{-1} protein 5 min^{-1}.

4.2.iv. Estimation of soluble protein

An acetone precipitation was carried out before the protein assay in order to remove interfering compounds such as phenols. A small volume of sample was shaken up with 25 volumes of ice cold acetone and the mixture centrifuged at high speed in an MSE Minor centrifuge to precipitate the protein. The acetone was discarded and the protein redissolved in a known volume of 2% sodium carbonate in 0.1M NaOH.

Protein was estimated using the Folin-Ciocalteau method of Lowry et al.(1951). A calibration curve was constructed using a freshly prepared solution of bovine serum albumin at concentrations of 10 to 100 μg $sample^{-1}$ (Appendix 4.1a). Results were expressed as mg protein ml^{-1}.

4.2.v Extraction of Alliinase

10g tissue were homogenised using a pestle and mortar with 10ml 0.2M potassium phosphate buffer, pH 6.8, in 0.3M sucrose for two minutes, then filtered through five layers of muslin. The filtrate was centrifuged for 15 minutes at

3,500 x g in an MSE Superspeed 65 centrifuge, and the supernatent re-centrifuged for 20 minutes at 30,000 x g to produce a clear yellow supernatent. The soluble proteins were precipitated from this supernatent by the addition of solid ammonium sulphate to 75% w/v saturation. After stirring for four hours at 4°C, the mixture was centrifuged for 20 minutes at 30,000 x g and the resulting pellet stored at -20°C for assay.

4.2.vi Assay for alliinase activity

This was a modification of the method of Schwimmer and Mazelis (1963) where synthetic S-propyl - L - cysteine sulphoxide was used as a substrate. Alliinase activity in each pellet was assayed at two protein concentrations of approximately 0.1 and 0.05 mg protein ml^{-1} reaction mixture. These concentrations were more accurately determined by the method of Lowry et al. (1951).

The reaction mixture contained 0.4ml 50mM S-propyl - L - cysteine sulphoxide, 0.1ml 0.5mM pyridoxal phosphate, 0.4ml 0.1M sodium pyrophosphate buffer, pH 9.0 and 0.1ml sample. The mixture was incubated in a water bath at 25°C, 0.1ml samples removed at various times and the reaction stopped by the addition of 1ml 10% TCA. Each sample was made up to a total volume of 2ml and pyruvate estimated as described above.

4.3 Results

4.3.i Induction of Differentiating Systems

Plate 4.1a shows the appearance of the roots obtained from onion callus cultures only two weeks after transfer of undifferentiated callus to the appropriate medium. Roots were formed mainly from the surface of the callus which was in contact with the medium and consequently most roots grew down into the agar. The roots were of normal appearance with true root hairs. Since root production was routinely obtained, material was available for most analyses.

Shoot production as shown in Plate 4.1b was much more difficult to control. The only shoots obtained in this study were produced by callus on root-inducing medium. In some cases, shoots formed in the dark and later turned green when the flasks were placed under illumination, whilst in other cases shoots were only produced after the callus was placed in the light. Green areas were often formed on treated callus but only on one occasion was a sample of this green callus tissue separated by TLC - electrophoresis. Green shoot material was limited so only the amino acid analysis and separation by TLC - electrophoresis were carried out.

The length of time required to induce onion bulb sprouting (Plate 4.2) varied according to season, being as much as four weeks in some cases.

Germination of onion seeds sown on moist cotton wool occurred three or four days after sowing. One week after

Plate 4.1 Morphogenesis in onion callus cultures growing in the absence of 2,4-D a) root production, b) production of shoots and green callus areas.

a

b

Plate 4.2 Sprouting onion bulb used to provide root and leaf material for radioactive feeding experiments and amino acid analysis.

sowing, most of the seeds had germinated, many of them to the stage of shoot emergence. At this stage, the seedling was approximately 15mm long. Seedlings were sampled for analysis by TLC-electrophoresis and amino acid analysis three weeks after sowing at which time the second leaf was emerging and an extensive root system had formed. Plate 4.3 shows the various stages of seedling growth.

4.3.ii Estimatation of Enzymically Produced Pyruvate and of Alliinase Activity

The data obtained from the assays for pyruvate production and alliinase activity are presented in Table 4.1. It was shown that the level of pyruvate produced in callus tissue was only 0.22% of that in the dormant onion bulb, whilst the level in a sprouting bulb was significantly higher than that of the dormant tissue. The pyruvate level in callus roots was higher than in the undifferentiated callus tissue but it was much lower than the levels found in the roots of onion bulb or seedling. In both the bulb and the seedling, levels of pyruvate were higher in the shoots than the roots, whilst the levels found in the seedling organs were above those found in the corresponding organs of a sprouting bulb.

The level of alliinase activity was higher in callus than in dormant onion bulb tissue although they were of the same order of magnitude, whilst the level in active bulb tissue was increased in comparison with both of these tissues. The level of alliinase activity was much greater in root tissue than in the shoots of both seedling and sprouting bulb.

Plate 4.3 Stages of onion seedling development after germination on moist cotton wool at room temperature in a 16 hour photoperiod.

Table 4.1 Levels of enzymically produced pyruvate and of alliinase activity in various types of differentiating tissue.

Sample	Pyruvate (μ moles g^{-1} fresh weight)	Alliinase (μ moles Pyruvate mg^{-1} protein)
Dormant onion bulb	9.02	1.62
Sprouting onion bulb	14.99	14.00
Callus tissue	0.02	3.92
Bulb roots	11.34	14.16
Seedling roots	24.73	31.51
Callus roots	1.34	-
Root producing callus	-	-
Bulb shoots	18.03	7.35
Seedling shoots	27.16	6.33
Callus shoots	-	-

The activity in the seedling shoots was at the same level as that found in bulb leaves, whilst the activity in seedling roots was more than twice the level in the bulb roots.

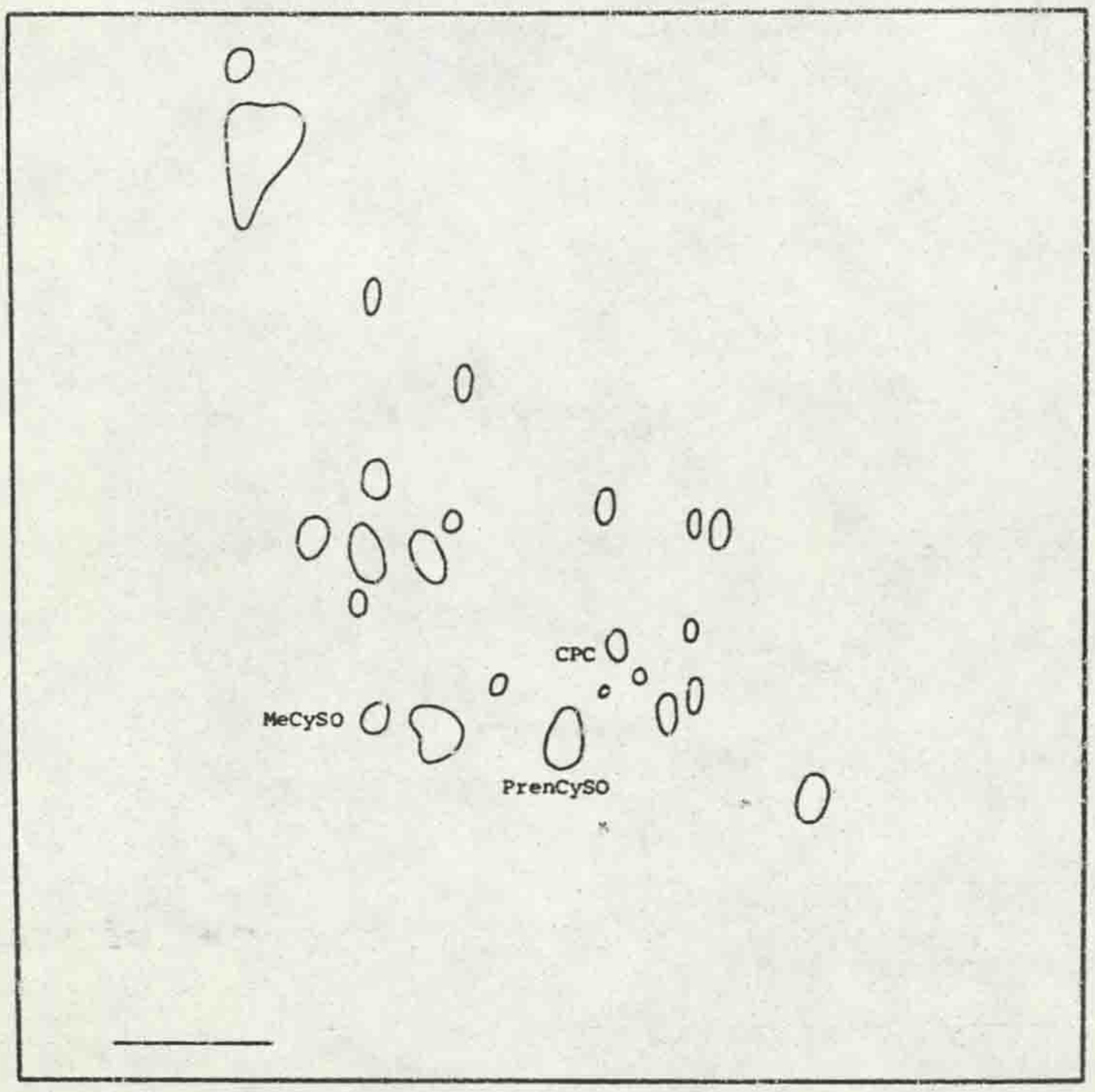
Crushing the callus-produced root tissue resulted in the generation of onion odour of an intensity approaching that of fresh onion, whilst shoots regenerated from callus had a faint onion smell which had no lachrymatory effect. A lachrymatory effect was detected in the crushed root material although the effect was slower than that observed when onion bulb tissue is damaged. The presence of only a faint onion smell in the shoot material may have been due to dilution effects since cultured tissues are known to have a higher water content than the corresponding tissues of the intact plant. Similarly, there may not have been sufficient amounts of flavour compound present to produce a significant organoleptic effect.

4.3.iii TLC-Electrophoresis Data

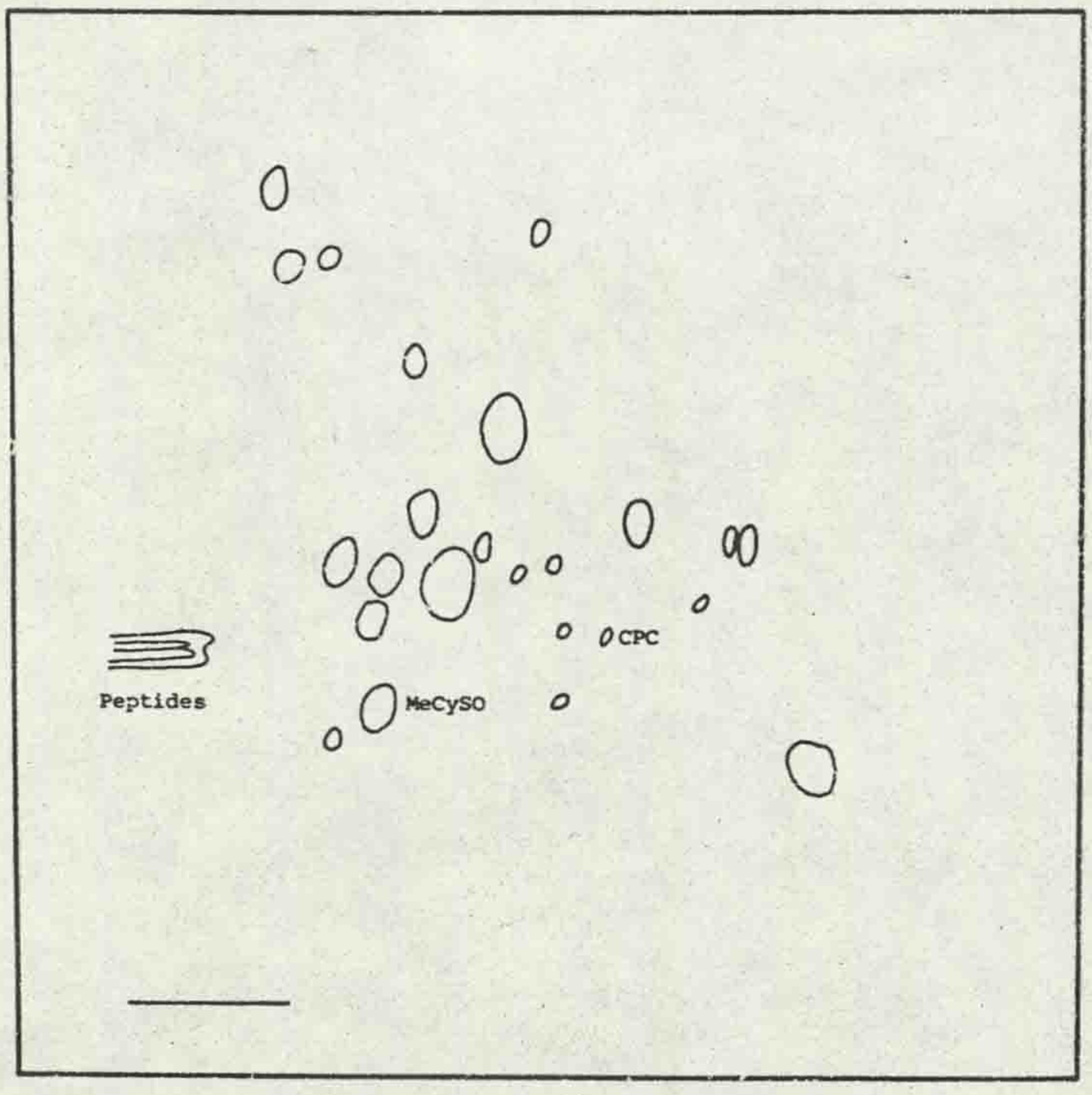
Figures 4.1a and 4.1b show the amino acid distribution of onion and callus extracts respectively. The pattern of free amino acids is similar in the two extracts but it must be remembered that three to four times the amount of callus extract was applied to each TLC plate in comparison with the onion extract. Therefore, there are obviously differences in the levels of each component in the extracts, However, for this investigation, the presence or absence of a particular compound was more important. Differences in the amounts of flavour compounds in callus and onion bulb extracts will be discussed later.

Fig. 4.1 Thin layer chromatogram of amino acid extracts of a) onion bulb tissue, b) onion callus material.

a



b

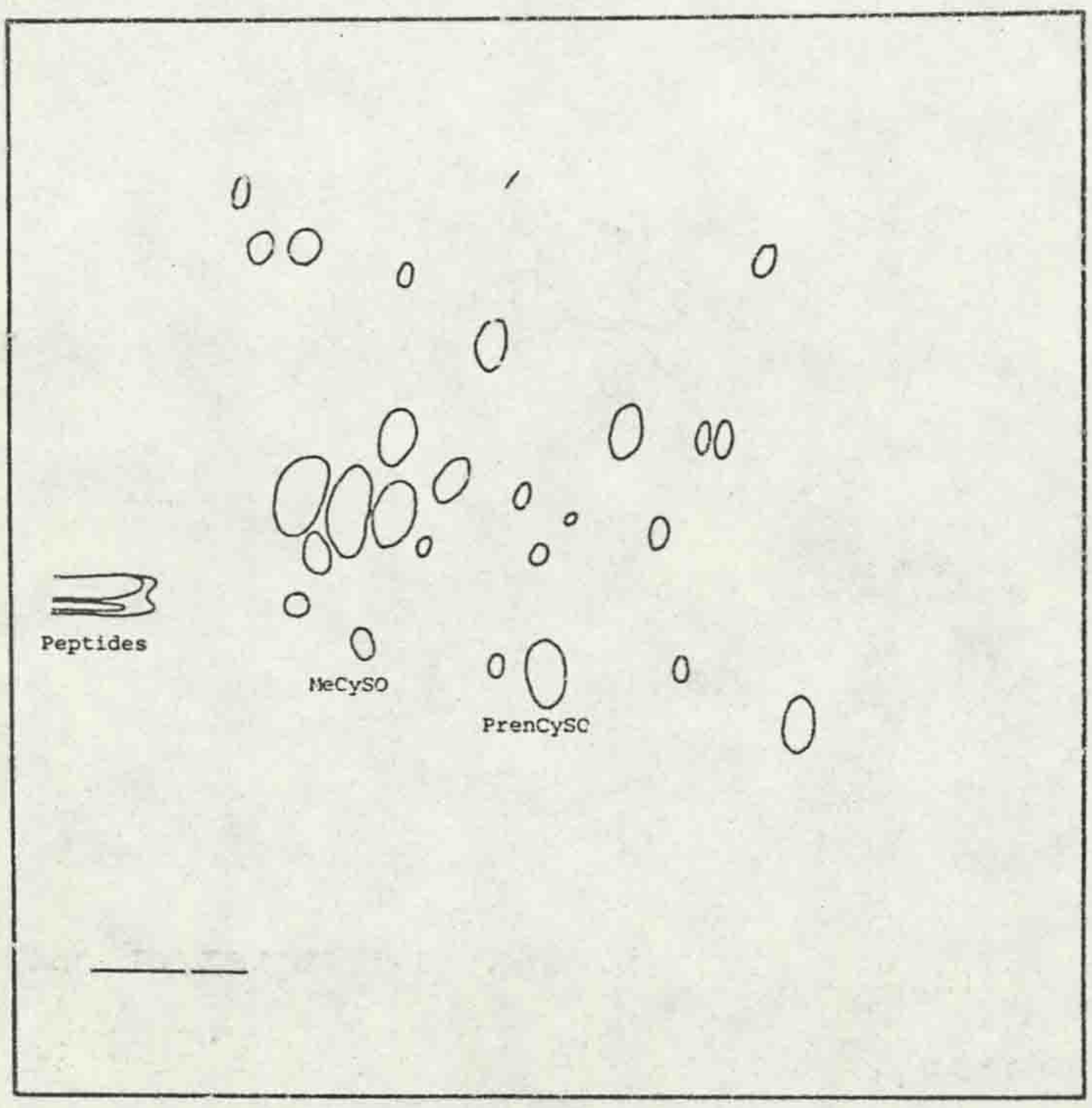


As already noted in Chapter 3, the most obvious difference between the two tissues was the lack of flavour compounds in the callus. Particularly striking was the absence of Pren Cy SO in the callus extract. However, Fig. 4.2a shows that in an extract of callus-produced roots, Pren Cy SO was present. A similar pattern was seen in an extract of callus shoots (Fig. 4.2b). Other unidentified flavour compounds were present which were not found in undifferentiated callus. When the patterns obtained from callus roots and shoots were compared with those from roots and shoots of the onion bulb and seedling, they were found to be almost identical (Figures 4.3a - 4.4b). Similarly, the amino acid distribution of a sprouting onion bulb (Fig. 4.5a) was the same as that of a dormant bulb. It was interesting to note that an extract of differentiated callus which had given rise to roots produced the same pattern of amino acids (Fig. 4.5b) as that obtained from an extract of the roots themselves. The only difference in amino acid composition as determined by TLC-electrophoresis was the presence of peptides in undifferentiated and root-forming callus, and in the roots and shoots derived from callus and sprouting bulb. No peptides were detected in extracts of dormant or sprouting onion bulb tissues, seedling roots or shoots.

Extracts were prepared from seedlings at various stages of development, including an extract of dry seeds. Separation of these extracts by TLC-electrophoresis showed that the flavour compounds of onion and Pren Cy SO in particular were present at all stages of growth. The amino acid pattern obtained from

Fig. 4.2 Thin layer chromatogram of extracts from a) callus-produced roots, b) shoots regenerated from callus tissue.

a



b

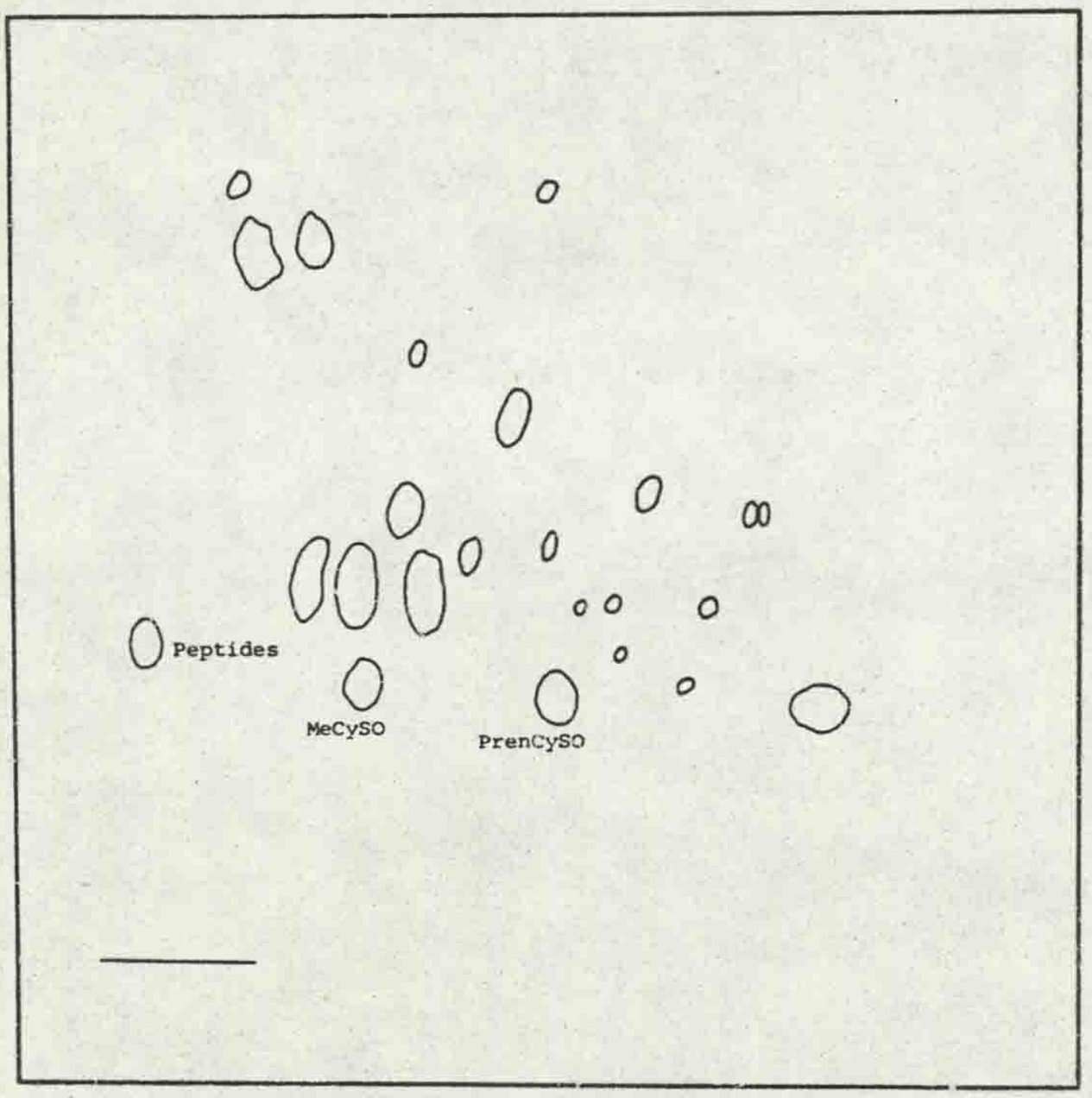
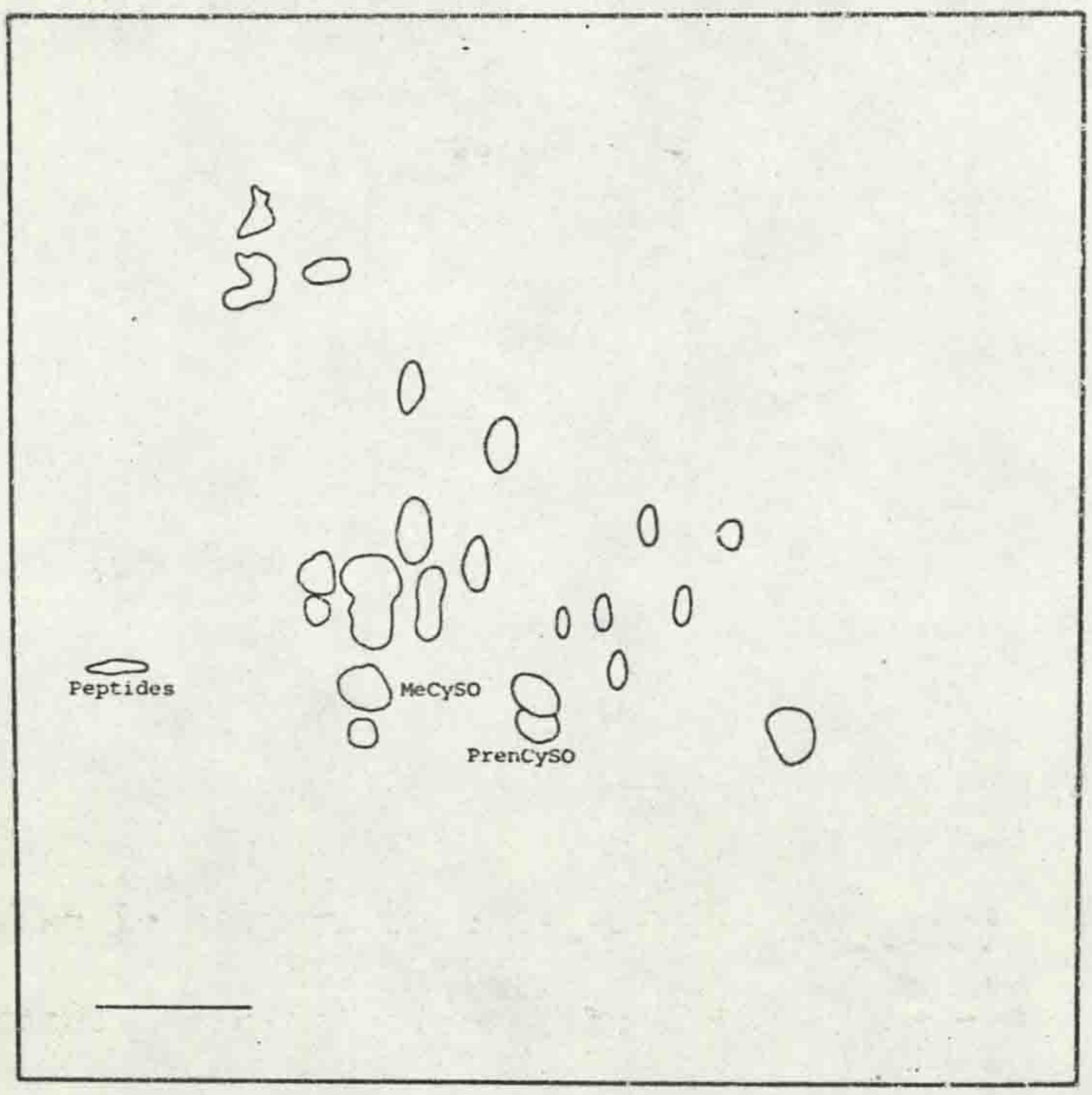


Fig. 4.3 Thin layer chromatogram of extracts from the roots of a) onion bulb, b) onion seedling.

a



b

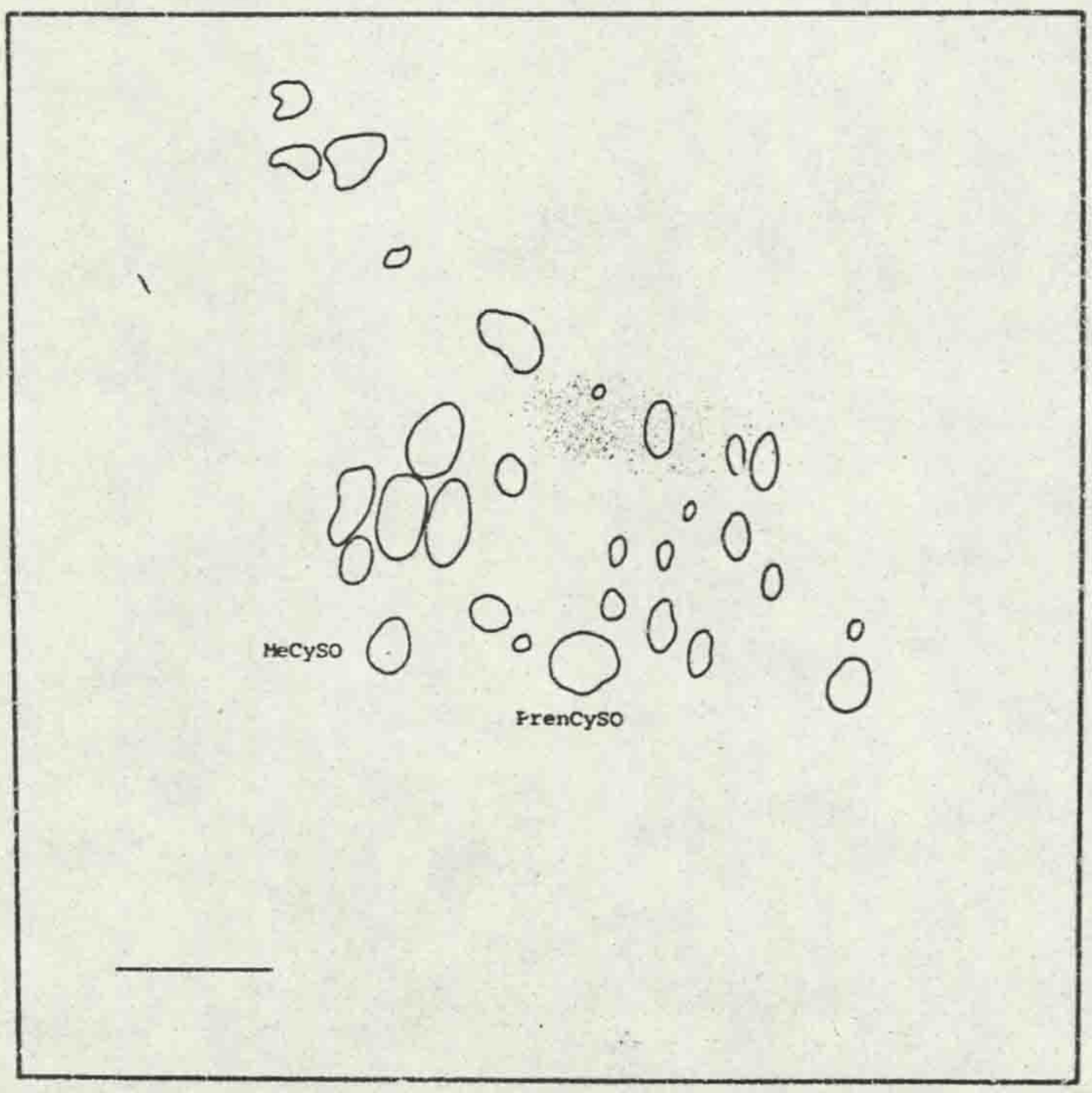
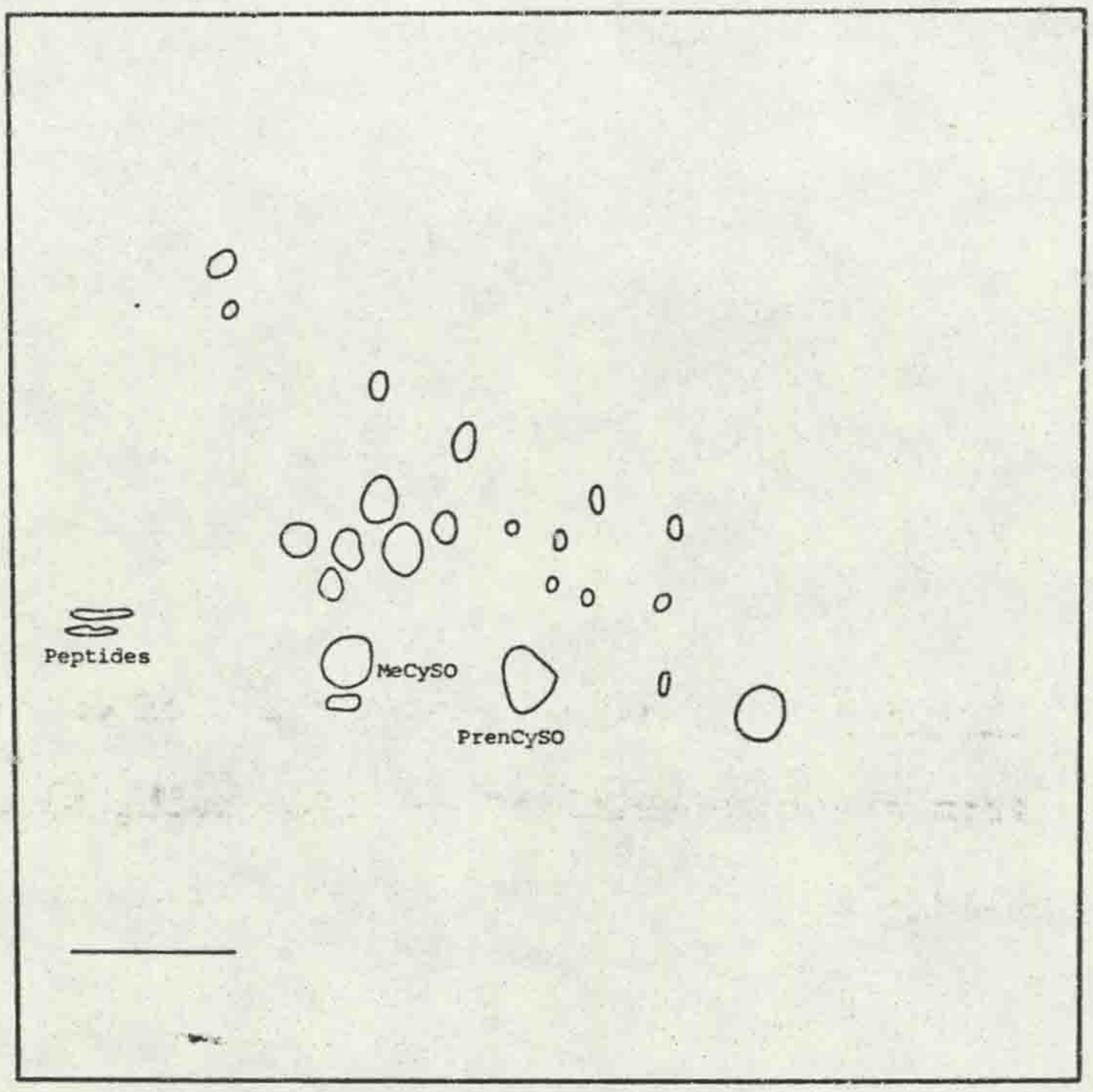


Fig. 4.4 Thin layer chromatogram of extracts from shoot material of a) onion bulb, b) onion seedling.

a



b

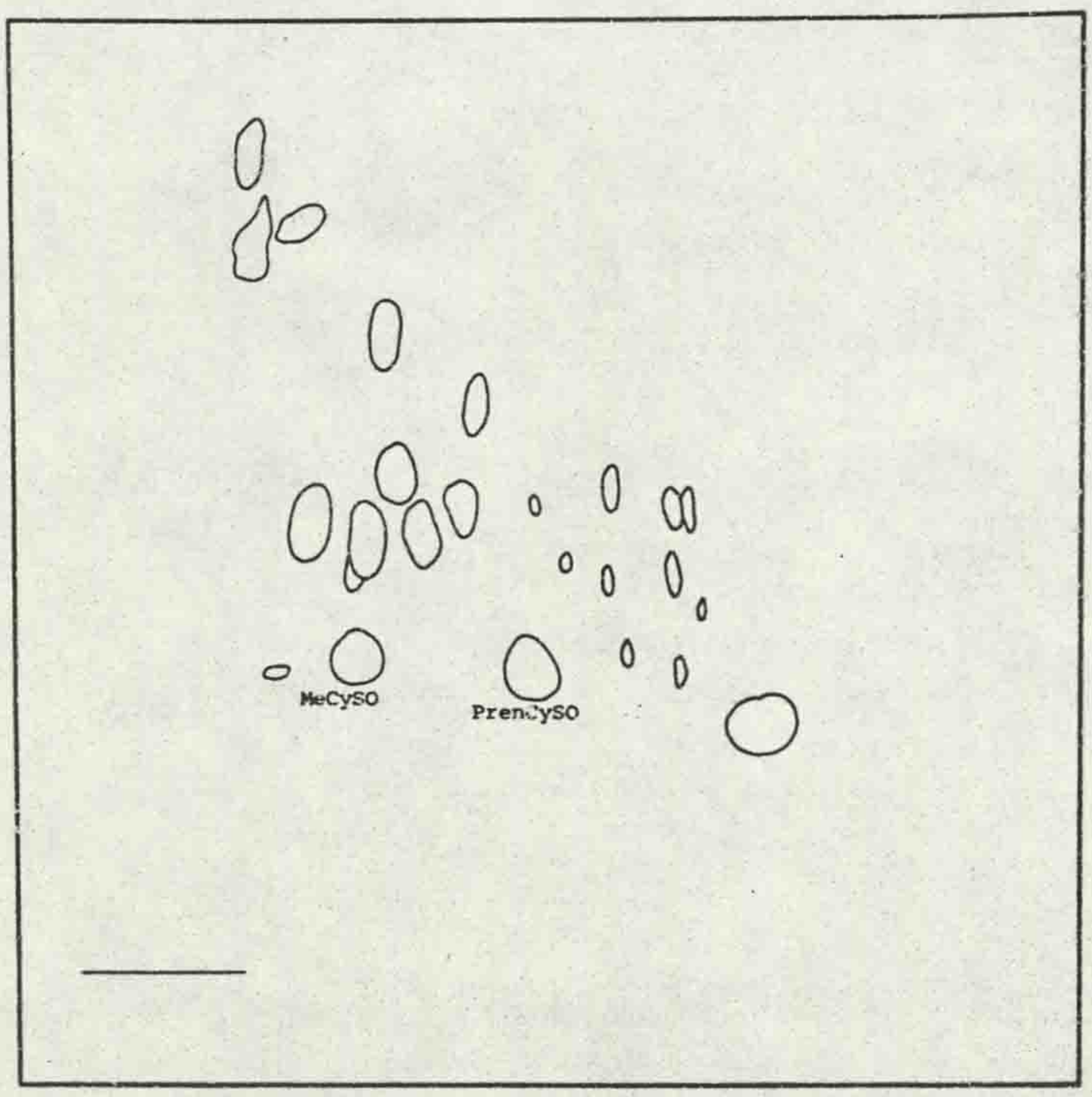
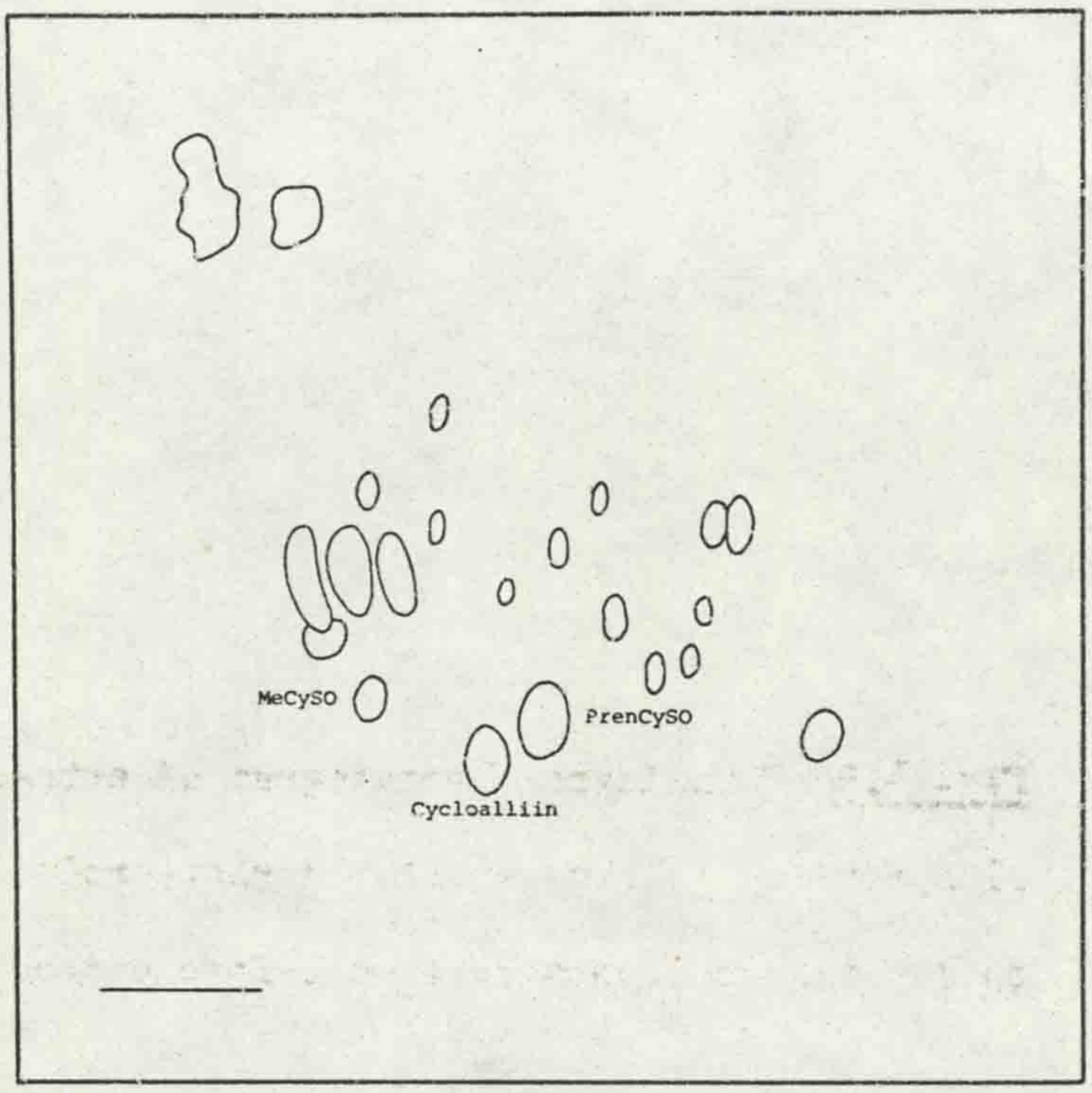
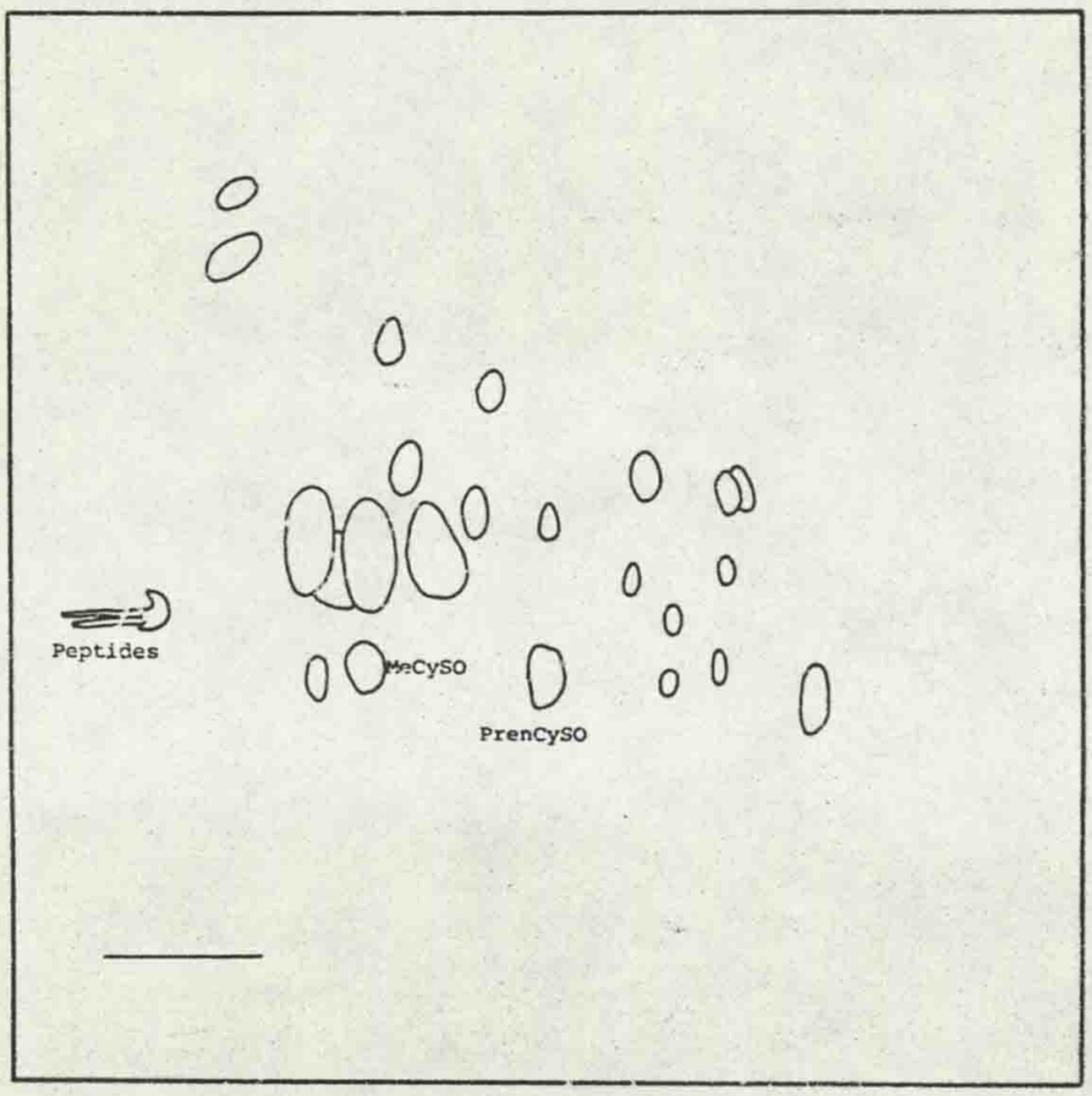


Fig. 4.5 Thin layer chromatogram of extracts from a) sprouting bulb material, b) root-producing callus tissue (roots removed before extraction).

a



b



each of these extracts was identical to those of the seedling roots and shoots shown in Figure 4.3b and 4.4b respectively.

The presence of the flavour compounds in callus-produced roots and shoots was confirmed by amino acid analysis. However, the method of extraction was not rigorous enough to provide quantitative data so the technique was used only as a means of identification of the flavour precursors. Preliminary investigation of extracts of onion bulb and callus tissue showed that, in general, the levels of free amino acids in callus extracts were much lower than in extracts of onion bulb tissue (Table 4.2). This was reflected in the amount of extract required to produce equivalent TLC - electrophoresis separations of the two extracts.

Me Cy SO was present in both extracts but was more abundant in callus, whilst Pren Cy SO, which was absent from callus, was present in large amounts in the onion. Only trace amounts of CPC were found in an onion extract but larger quantities were detected in callus. This was to be expected since the biosynthetic chain operates very slowly in callus whilst in onion tissue the pathway is more active and so no accumulation of intermediates, such as CPC, will occur.

Table 4.2 Levels of free amino acids and flavour compounds in onion bulb and callus tissues.

Amino acid	μ mole g^{-1} fresh weight	
	Onion	Callus
Cysteic acid	1.69	
Lysine	0.68	0.30
Histidine	0.19	0.27
Arginine	5.08	0.20
Me Cy SO	6.40	16.60
Pren Cy SO	12.53	
Aspartic acid	2.36	1.95
Threonine/Asparagine	6.99	
Serine	Present	
Glutamic acid	0.89	2.06
CPC	Trace	1.41
Glycine	0.51	0.07
Alanine	0.08	0.16
Valine	0.16	0.27
Isoleucine	0.14	0.05
Leucine	0.34	0.04
Tyrosine	0.24	0.07
Phenylalanine	Trace	
Proline	Trace	

4.4 Discussion

The level of pyruvate in callus was very low compared to that of onion bulb tissue, a fact reported by Selby and Collin (1976) who used similar methods to those described here, and also by Davey et al. (1974) and Freeman et al. (1974) who used sensory tests combined with gas chromatographic evidence as well as estimation of enzymically produced pyruvate. Freeman et al.(1974) also showed that the level of pyruvate increased in callus tissue on root initiation as reported here. Since enzymically produced pyruvate is a direct measure of the amount of flavour precursors in onion tissues, these results reflect those of simple sensory tests. From the sensory tests it was obvious that whilst rapidly growing, undifferentiated callus tissue produced only a faint onion smell, the odour intensity increased when the callus differentiated to form roots. However, this increased level of pyruvate in differentiated callus was still much lower than the amount found in normal roots, although the TLC pattern showed the presence of Pren Cy SO in callus-produced roots and also in the shoots regenerated from callus. Although shoot production was achieved earlier by Fridborg (1971) and Dunstan (1977), no investigation had been made regarding the flavour components of these tissues.

From the evidence presented here it can be shown that the pattern of flavour precursors in callus-produced roots and callus shoots are identical to the patterns obtained from normal roots and shoots respectively. This indicates that on differentiation, the biochemical pathways leading to flavour

precursor synthesis were regenerated in the callus. Thus, roots and shoots regenerated from callus tissue possess the same flavour precursors as those of naturally produced organs, although the levels of these compounds may differ.

The presence of Pren Cy SO in differentiated callus is important in that it may reflect a change in metabolism of the callus tissue itself when it regenerates roots and shoots, rather than changes occurring only in the regenerated, organized tissues. Preliminary investigation of shoot-producing callus indicated a similar situation to that found in rooting callus where Pren Cy SO was present in the callus tissue as well as the regenerated roots. However, this examination of shoot-forming callus requires more critical investigation to confirm these results. Presence of Pren Cy SO in root-forming callus may have been due to the presence of root initials remaining deep in the callus mass and microscopic analysis is required to show if this was the case. However, it is thought unlikely that the large spot representing Pren Cy SO on the TLC plate was the result of contamination by a relatively small number of root cells.

These results suggest that the changes occurring on callus differentiation to form organs occur in the callus tissue itself and not just in the regenerated root and shoot tissues. Thus, it would be of interest to follow the development of the metabolic pathways during the process of morphogenesis from the time callus was transferred to differentiating medium to the appearance of recognisable root and shoot initials. For example, it is possible that the metabolic changes arise as a result of altered levels of growth-promoting substances in the medium and thus they may precede any visible changes

in callus structure which occur on morphogenesis.

Levels of pyruvate were greater in sprouting bulb tissue than in the dormant bulb. Similar findings were reported by Bandyopadhyay and Tewari (1976) and were to be expected since it is known that much of the flavour complement of the dormant onion bulb is bound with glutamic acid to form γ-glutamyl peptides. Numerous such peptides have been discovered as typical components of Allium species (Virtanen 1965). Nine peptides were isolated from onion bulbs in pure form and were chemically characterised by Virtanen and Matikkala (1960, 1961). They included the bound forms of Me Cys, CPG and Pren Cy SO of which γ-glutamyl Pren Cy SO was reported to be the major component in the dormant onion (Matikkala and Virtanen 1967, Virtanen 1969). These peptides are not susceptible to alliinase action and so flavour precursors bound in this way are not available for flavour production. However, evidence has been presented (Matikkala and Virtanen 1965, Schwimmer and Austin 1971) to show that specific transpeptidase enzymes exist in sprouting bulb tissue which degrade the peptides and liberate Pren Cy SO or other alkyl cysteine sulphoxides. These compounds are then available for breakdown by alliinase to produce characteristic flavour compounds of onion.

γ-glutamyl peptidase activity was first reported to be present in sprouting onion bulbs by Matikkala and Virtanen (1965) whilst later work by Schwimmer and Austin (1971) and Austin and Schwimmer (1971) confirmed this report and described the isolation and characterization of the enzyme. They confirmed that the

enzyme was present in sprouted bulb tissue but absent from the dormant bulb and that sufficient activity was recorded in the former to account for the slow disappearance of γ-glutamyl peptides which accompanies bulb sprouting. Schwimmer (1971) was also able to demonstrate the coupled reaction which was involved in the breakdown of the peptides by transpeptidase enzymes followed by alliinase activity to release pyruvate and the volatile flavour compounds of onions. Thus, the results obtained in this investigation can be explained by the presence of transpeptidases in sprouting bulb tissue (Abraham et al. 1976) which render available larger amounts of flavour precursors for alliinase action than would be available in dormant tissue. As expected, no peptides were present in extracts of sprouting bulb. However, peptides were never detected in extracts of dormant bulb during this investigation although the reasons for this are not clear. Granroth (1970) was able to show incorporation of labelled sulphate into peptides in slices of dormant onion bulb but he failed to demonstrate conversion of labelled CPG to γ-glutamyl Pren Cy SO in the same material.

The presence of peptides was characteristic of callus tissue extracts. Since it is known that small amounts of flavour precursors are present in callus, it is possible that these compounds are present in the bound form as peptides and that no onion flavour is apparent in callus tissue because the callus lacks the transpeptidase enzymes required to release free alkylcysteine sulphoxides. Assays for the transpeptidase enzymes could be carried out using crude

homogenates of callus and sprouting bulb material to determine any differences in enzyme activity between the two tissues. It would also be of interest to investigate the transpeptidase enzymes of differentiated callus since this tissue appears to parallel that of the sprouting bulb with respect to the peptides present.

More pyruvate was found in shoot material than in the roots or sprouting bulb tissue. Similar results were reported by Bandyopadhyay and Tewari (1976) where they demonstrated the increasing levels of lachrymator precursor (Pren Cy SO) in onion bulb shoots and sprouting bulb material, which occurred as sprouting progressed. The amounts in the shoots were in excess of those in the bulb whilst the levels in both of these tissues exceeded those found in an unsprouted bulb. It was suggested that the high levels of precursor in the shoots were due to release of this compound from the bulb and subsequent translocation to the bulb sprouts. However, this does not explain the results described here where it was found that peptides were present in extracts of bulb roots and shoots.

Homogenates prepared from germinating seeds of Allium species were found to possess γ-glutamyl peptidase activity (Virtanen 1965) but it is uncertain if peptides were present in the seed extracts separated by TLC - electrophoresis here. In this study, no peptides were detected in seedling roots or shoots, so the differentiating systems of a sprouting bulb and germinating seedlings were not identical. From the results of these experiments it seems that

differentiating callus most closely parallels the situation in a sprouting bulb. If it is assumed that the callus lacks the necessary transpeptidase enzymes then it would appear that during differentiation, 'de novo' synthesis of flavour precursors takes place, rather than just mobilisation and translocation of available precursors to the leaves. In fact, it is likely that this also occurs in sprouting bulb shoots where some of the flavour precursor may be translocated from the bulb whilst the rest is produced by 'de novo' synthesis in the leaves.

It was thought that production of flavour precursors may have been connected with cellular organization and chlorophyll synthesis, since an extract of green callus areas produced an identical amino acid pattern to that of callus-produced shoots and normal shoots. However, a similar pattern was also produced from callus roots and root-producing callus extracts so any connection between chlorophyll synthesis and flavour production is uncertain. Quantitative data are required to clarify the situation.

The level of alliinase activity in callus was found to be of the same order of magnitude as in the dormant bulb. This further confirms the work by Freeman et al. (1974) and Selby and Collin (1976) who presented evidence to show that the absence of onion flavour in callus tissue was due to absence of flavour precursors rather than a reduction in alliinase activity.

Extracts of seedling roots and shoots at various stages of development showed that the flavour compounds were present at all

stages of growth including the dry seed, although it was difficult to identify some of the compounds in the seed extract due to unsuccessful separation by TLC - electrophoresis. To confirm the presence of individual compounds the seed extract must be purified before use to remove all interfering compounds. However, these results were in agreement with those of Becker and Schuphan (1975) who showed that the flavour compounds were associated with vascular tissue and therefore they were present at all stages of growth. In the present experiment, the first sample of the germinating seed was taken after the cells had begun to organise into tissue types so all samples showed the presence of flavour precursors. In the work reported by Becker and Schuphan (1975) evidence was obtained by histochemical methods. However, the staining techniques were not specific to the flavour precursors and so more sophisticated methods are required to provide further support for their findings.

Although some flavour precursors may have been present in the dormant seed, further synthesis of these compounds must begin at some later stage in development and from the available evidence it is likely that this will be during the initial organization of vascular tissue. Therefore, samples of the seed, separated into its various components, need to be tested at several early stages in germination in order to determine the exact point of 'de novo' synthesis of flavour precursors. However, this may prove to be a difficult task due to the small size of the seed and the toughness of its testa.

CHAPTER 5

FINE STRUCTURE OF ONION AND CALLUS CELLS.

5.1 Introduction

The most obvious biochemical difference between the tissues of onion and callus was the absence of flavour production in callus. During differentiation however, flavour production increases (Chapter 4) which suggests that structural differences between the cells may contribute to the difference in biochemical capacity. In recent years, many investigations have been carried out on the ultrastructure of cells in culture (Halperin and Jensen 1967, Sutton-Jones and Street 1968, Yeoman 1970, Yeoman and Street 1973, Wilson et al. 1974) but these studies have been concerned mainly with developmental changes as callus or suspension cultures are formed from previously quiescent tissue. The absence of any comparative studies between intact plant and tissue cultures may be due to the fact that cultured cells do not correspond directly with any particular tissue of the plant (Street 1969). For instance, cells of onion callus tissue have been examined by Davey et al. (1974) but no comparison with tissues of the plant body was made.

The purpose of this investigation was to establish the presence or absence of structural features in onion and callus cells which could be related to the difference in flavour production. Every cell of the onion plant produces flavour compounds and so for the purpose of this investigation callus

cells could be compared with cells from any part of the plant. In this case, bulb tissue was used. A comparison was also made between old, established callus and newly initiated callus since the morphology and growth rates of these two types showed considerable variation.

5.2 Materials and Methods

5.2.i Plant Material

Storage tissue was removed from the inner part of the bulb but did not include the central meristematic area which was to form the following year's shoot growth. Two types of callus were selected of which one was a clone of well established callus which had been cultured for approximately five years and the other, a newly initiated clone which had been cultured for a period of six months. Preliminary light microscope studies of the callus showed some variation in cell type. In selecting callus pieces for the electron microscope, an attempt was made to remove representative samples which would show this variation. Plate 5.1 shows the difference in morphology of the two callus types, the older one being more friable. The newly initiated callus formed a compact, nodular mass with a layer of mucilage over the tissue surface.

5.2.ii Preparation of Material for Electron Microscopy

Material was fixed in Karnovsky's fixative modified to contain 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH 7.2) for 5 hours, washed overnight in 0.1M cacodylate buffer then post fixed for 4 hours in 1% osmium tetroxide in sodium cacodylate buffer (pH 7.2). After two washes in the cacodylate buffer over one hour, the tissue was dehydrated in a graded series of ethanol. The tissue was then embedded in resin (Spurr 1969).

Sections were cut on an OMU3 Reichart Ultramicrotome at a thickness of 90nm. Sections were stained in a saturated solution of uranyl acetate in 50% ethanol for 30 minutes followed by 5 minutes in 1% lead citrate (Reynolds 1963). Sections were collected on 300 mesh uncoated copper grids and examined in a Kratos AEI Corinth 500 electron microscope operating at 60KV.

Plate 5.1 Comparison of compact, nodular, newly-initiated callus material with the softer, friable tissue of established callus.

5.3 Results

The main differences observed between onion and callus cells were comparable to those differences between quiescent and meristematic higher plant cells. In general, the fine structure of the onion cell was characteristic of a quiescent cell and some of the main features can be seen in Plate 5.2a. The cells were very large and thin-walled with large central vacuoles, some of which were apparently empty whilst others were filled with a fibrillar material of unknown composition. The cytoplasm formed a thin, peripheral lining along the cell wall and contained a minimal number of organelles such as ribosomes, mitochondria, dictyosomes and a small amount of endoplasmic reticulum. Mitochondria were spherical or rod-shaped in appearance (Plate 5.2b) and the dictyosomes were small. The endoplasmic reticulum was mainly smooth ER, ribosomes were usually free in the cytoplasm and polysomes were scarce. The flattened, peripheral nucleus was seen to contain a prominent nucleolus in which two distinct regions, the fibrillar and granular regions, were visible (Plate 5.3a). Many of the organelles were concentrated in the area of cytoplasmic thickening around the nucleus. No plasmodesmata were observed in any of these cells. In some cases (Plate 5.2b) the plasma-lemma was highly invaginated so that a space was formed between the plasma-lemma and the cell wall.

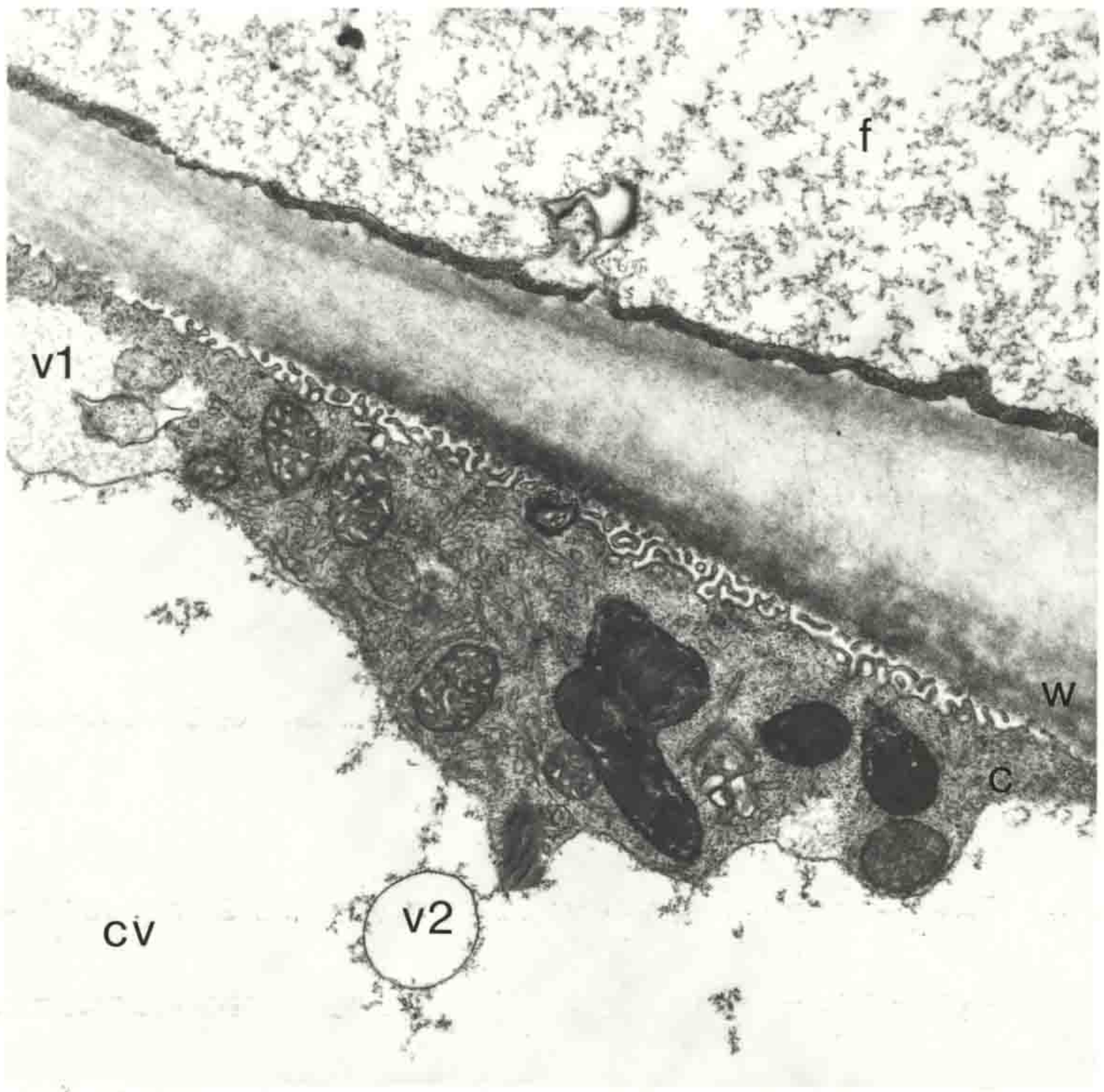
The distinctive feature of the onion cells was the presence of numerous small vesicles in the cytoplasm and in the central vacuole (Plate 5.3b). These were of two main types, of which the first was formed by an outgrowth of the cytoplasm into the central vacuole but was separated from the vacuole by the tonoplast (Plate 5.4a). This type of vesicle was always filled with a fibrillar type of

Plate 5.2 Electron micrograph of onion bulb cells showing:

a) large central vacuole (cv) which may contain some fibrillar material (f), thin cytoplasmic layer (c) lining the cell wall (w) and the vesicles found in the cytoplasm (v_1) and the central vacuole(v2). (x5,000).

b) rounded mitochondria, smooth endoplasmic reticulum (ER) and invaginated plasma membrane (pm). (x30,000).

a



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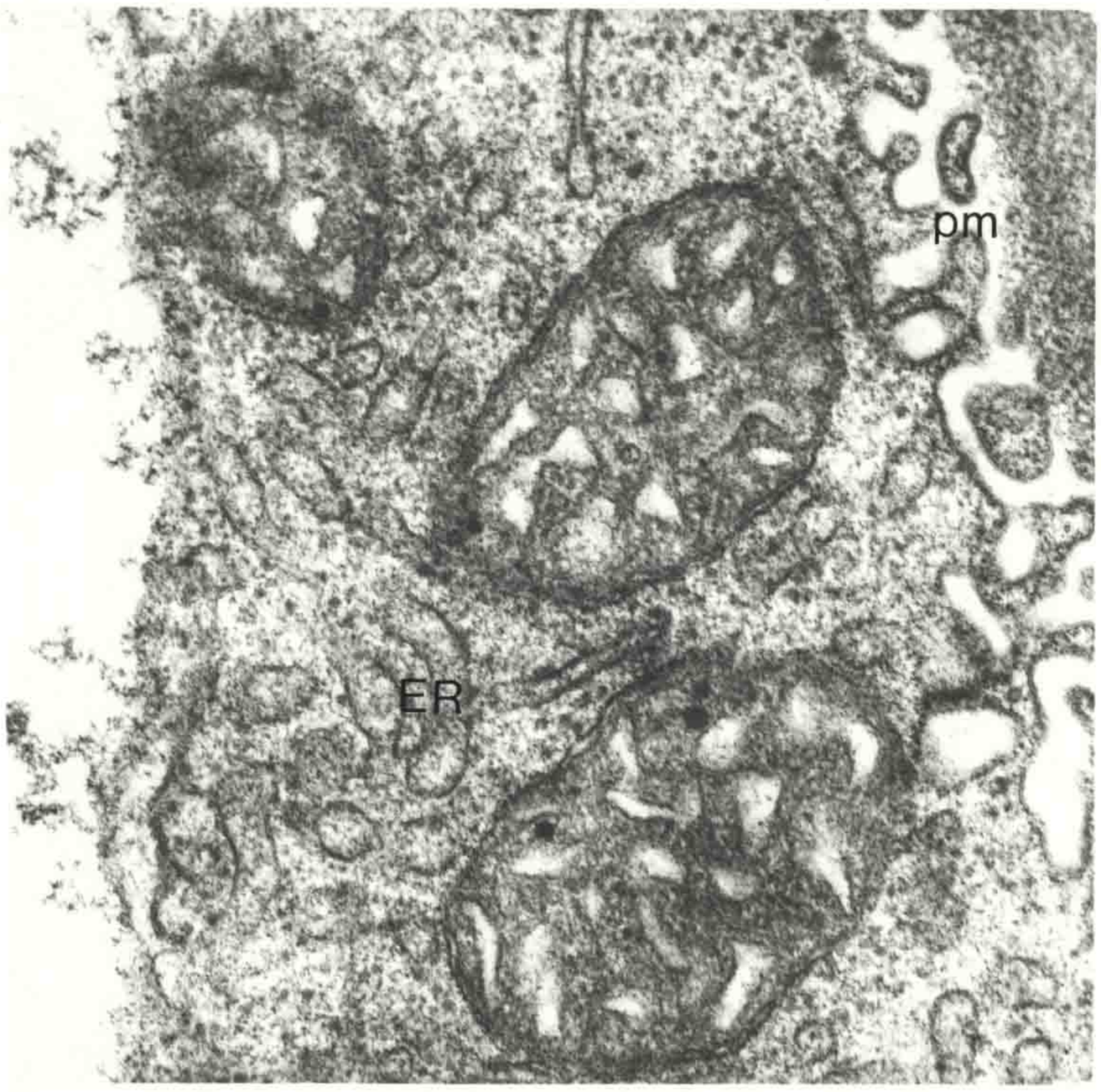
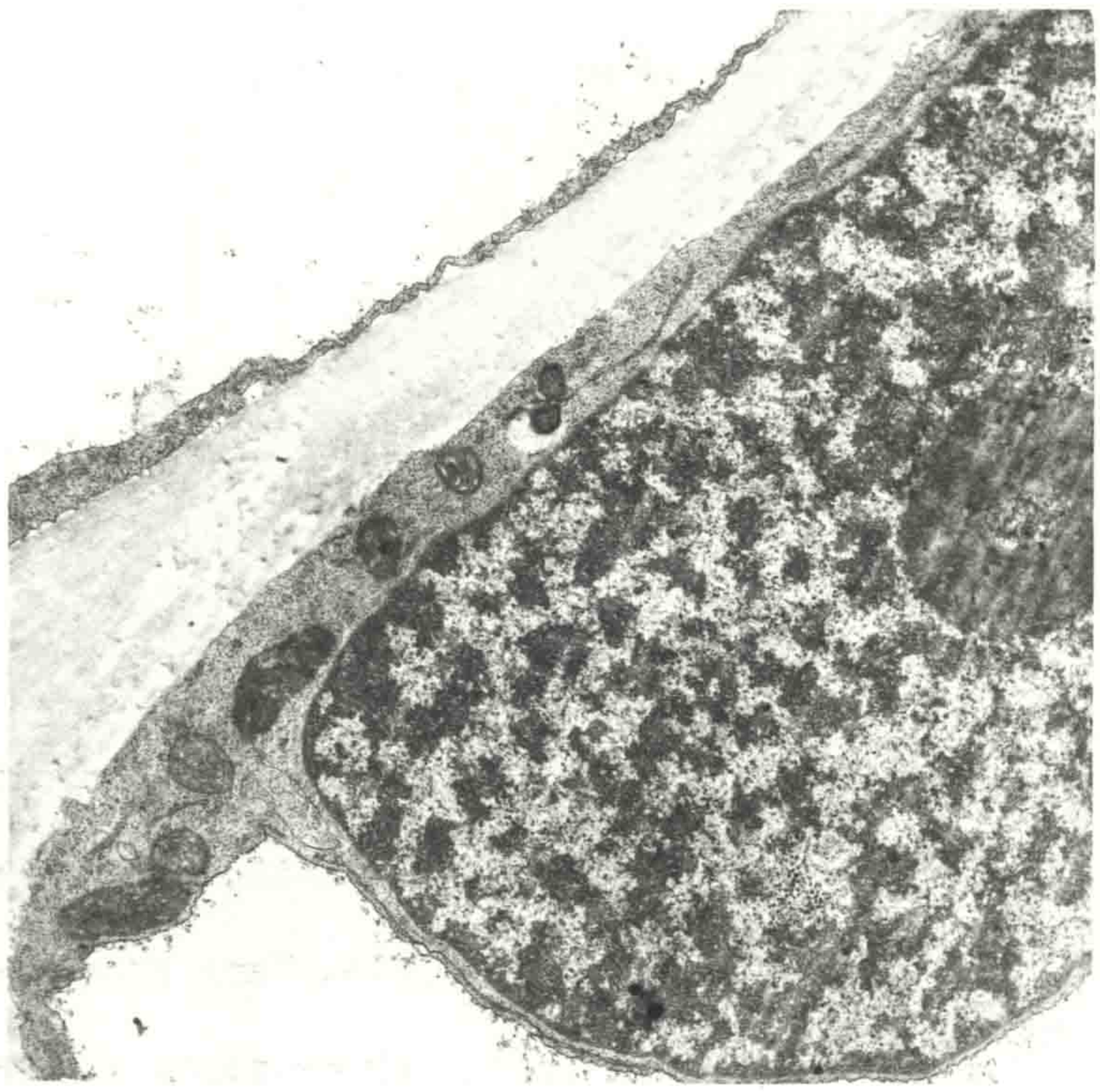


Plate 5.3 Section of onion bulb cell:

a) with flattened, peripheral nucelus containing a prominent nucleolus of which the fibrous and granular regions are clearly visible. Many other cell inclusions are concentrated in the cytoplasmic thickening around the nucleus. (x5,000).

b) containing two types of vesicle, one of which (v1) is an outgrowth of the cytoplasm which is separated from the central vacuole by the tonoplast and which contains fibrillar material. The second type of vesicle (v2) consists of a single-membrane bound structure which occasionally contains similar material. (x20,000).

a



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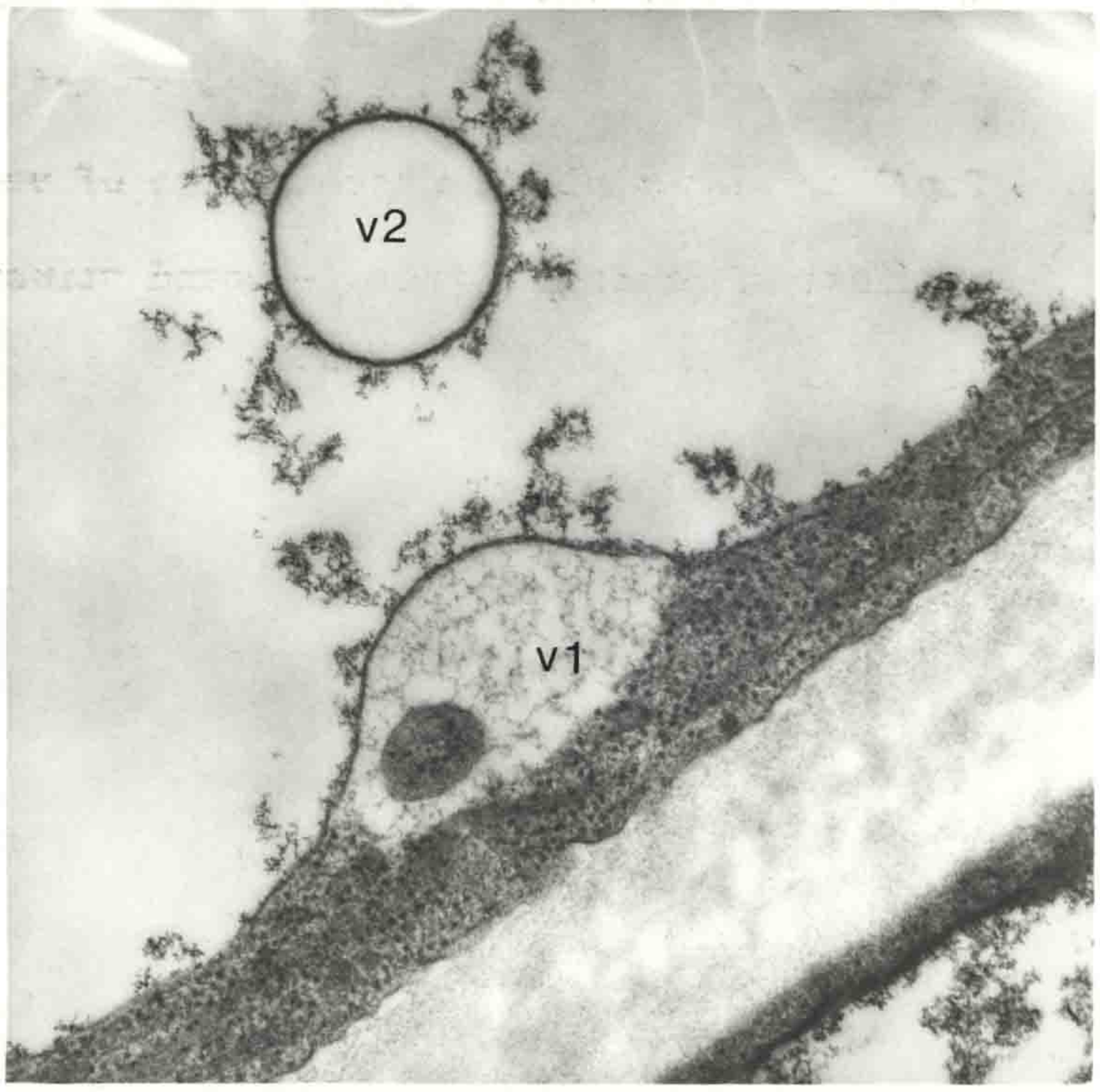
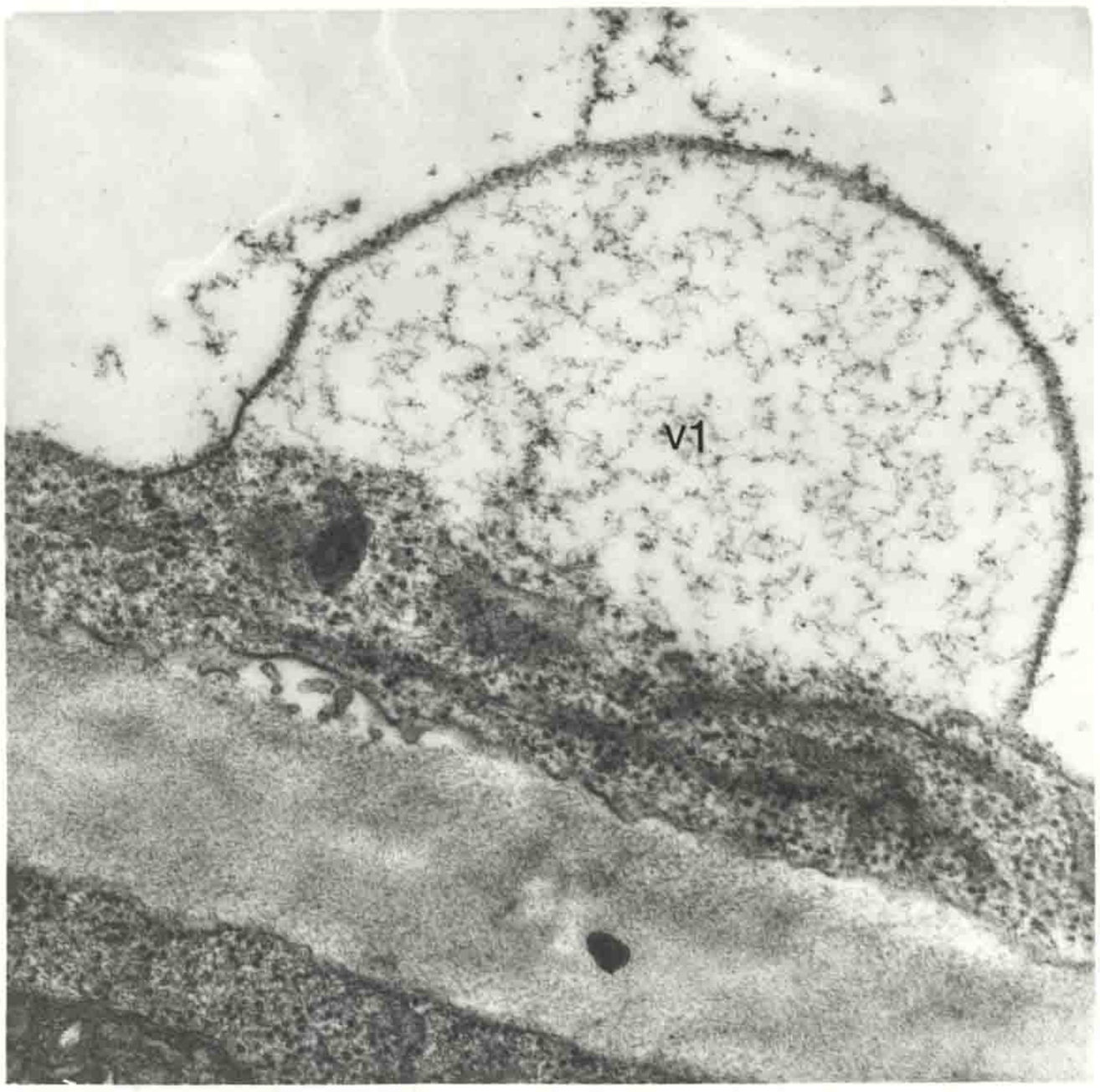


Plate 5.4 Section of onion bulb cell:

a) specialised vesicle (v1) found only in the onion cell, bounded by the cytoplasm and the tonoplast and containing a loose, fibrillar material of unknown composition. (x 20,000).

b) the second type of vesicle (v2) found in onion cells containing some fibrillar material. Often, these single-membrane bound vesicles are empty. (x12,000).

a



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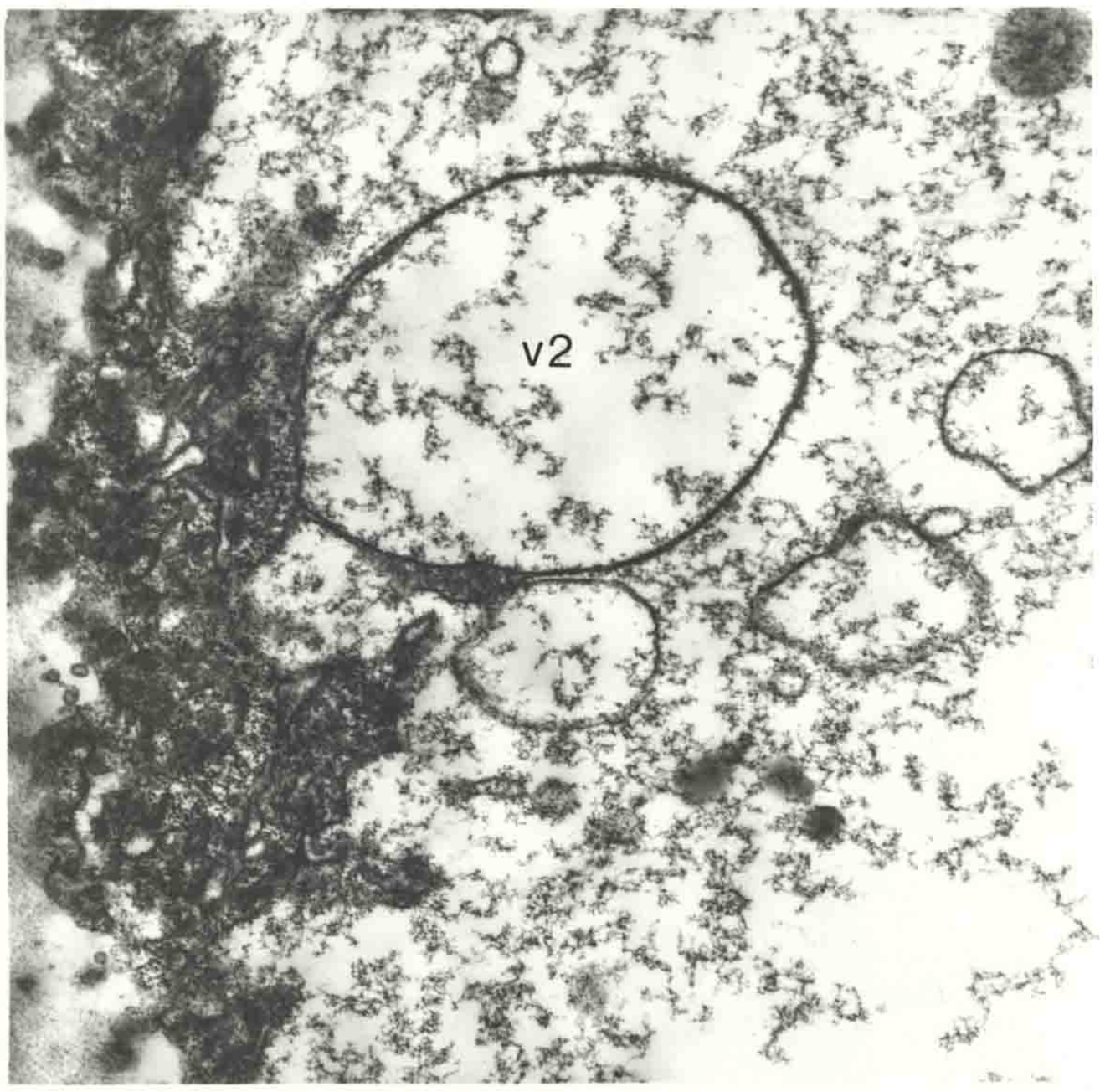


material. The second type was a single membrane bound vesicle found in the cytoplasm and the central vacuole (Plate 5.4b). Some contained a similar type of material to that described above, but many were empty. Also present in the onion cells were multi-vesicular bodies which consisted of double membrane bound vesicles containing numerous other vesicles of similar structure (Plate 5.5a). Some contained a loose, fibrillar type of material whilst others contained more dense deposits which may have been storage products.

In contrast, callus cells were characteristic of meristematic cells in that they were tightly packed cells with no intercellular spaces (Plate 5.5b). They had an increased volume of dense cytoplasm, prominent nuclei and several small vacuoles instead of a large central vacuole. Many of these vacuoles contained the fibrillar material described above. Callus cells were much smaller than those of onion and many of the thin walled cells were interconnected by plasmodesmata (Plate 5.6a). Occasionally, extremely thick walled cells were observed. The nucleus, positioned in the centre of the cell, was very large and conspicuous with a prominent nucleolus. The nucleus was usually irregular in shape with deep invaginations of the nuclear membrane producing a lobed appearance. Dense aggregations of chromatin were frequently observed.

Mitochondria existed in a variety of shapes and sizes and were numerous throughout the cytoplasm. Some were extremely elongated and many appeared to be dividing (Plate 5.6b). Cup shaped mitochondria were often observed. Dictyosomes were more abundant and larger than those of onion. They were always flat rather than curved and associated with numerous vesicles of assorted sizes (Plate 5.7a). Occasionally, groups of two or three dictyosomes were found. A prominent feature of callus cells was the abundance of

Plate 5.5

a) Section of onion bulb cell with complex, double-membrane bound vesicles and multi-vesicular bodies. (x5,000).

b) Electron micrograph of established callus tissue showing small, tightly-packed cells with dense cytoplasm, prominent nuclei, few small vacuoles and no intercellular spaces. The irregularly-shaped, centrally situated nucleus contains a prominent nucleolus with clearly distinguishable granular and fibrous regions and dense aggregates of chromatin. Plasmodesmata interconnect the thin-walled cells. (x2,000).

a

b

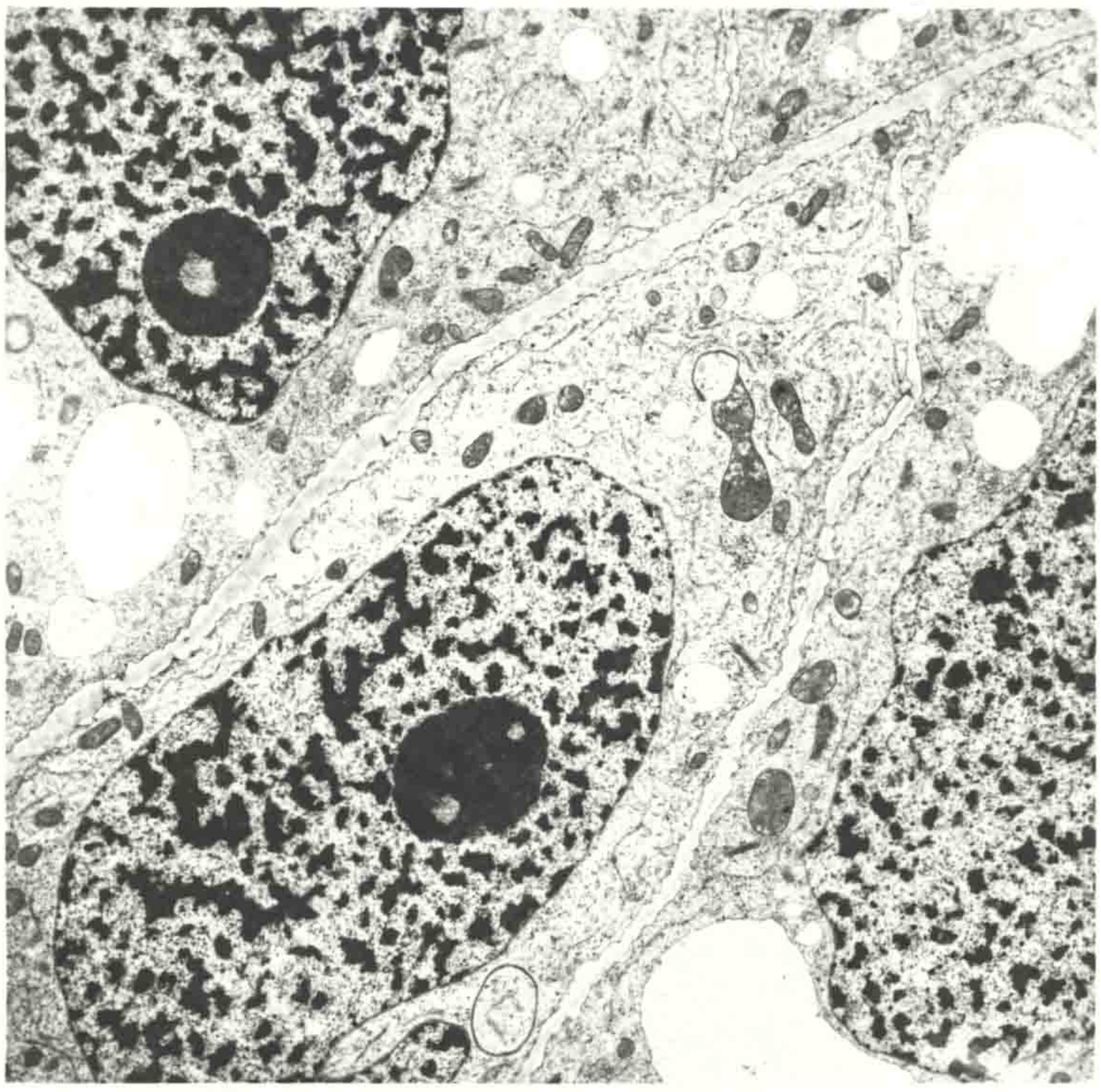
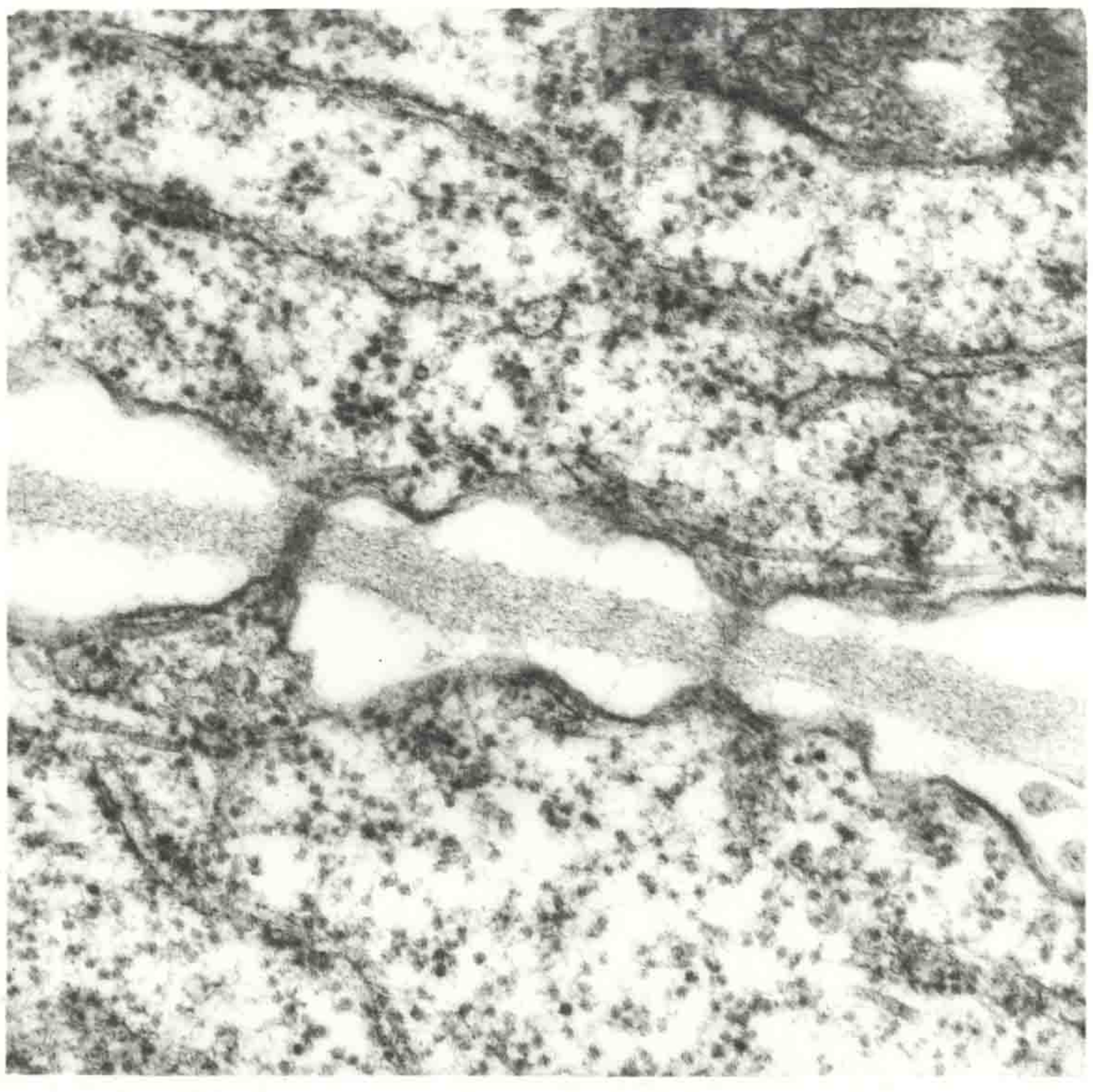


Plate 5.6 Section of established callus tissue with:

a) plasmodesmata connecting adjacent cells and abundance of ribosomes free in the cytoplasm or bound with membranes to form rough ER. (x30,000).

b) irregular and cup-shaped mitochondria, some of which are in the process of division. (x12,000).

a



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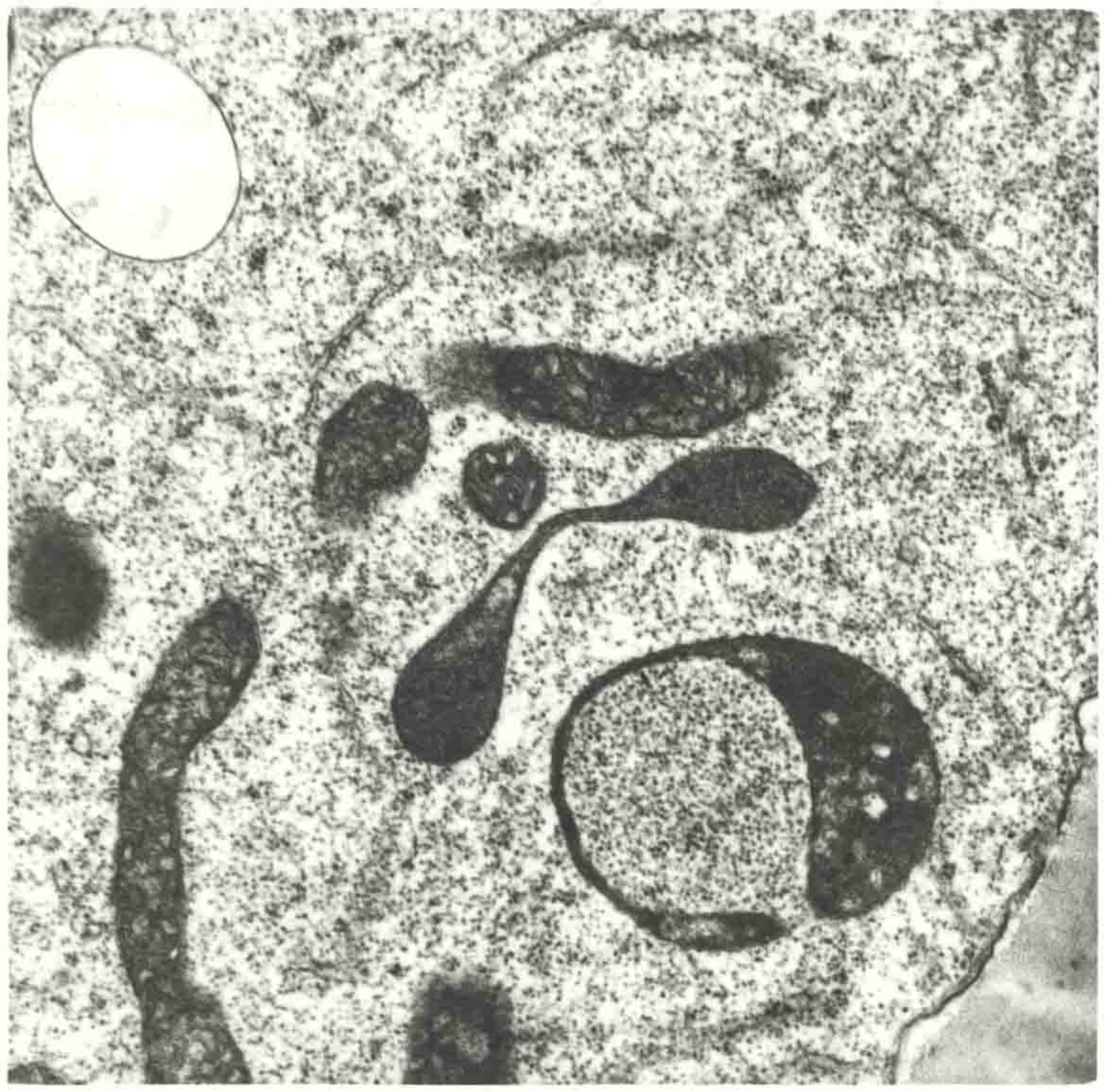
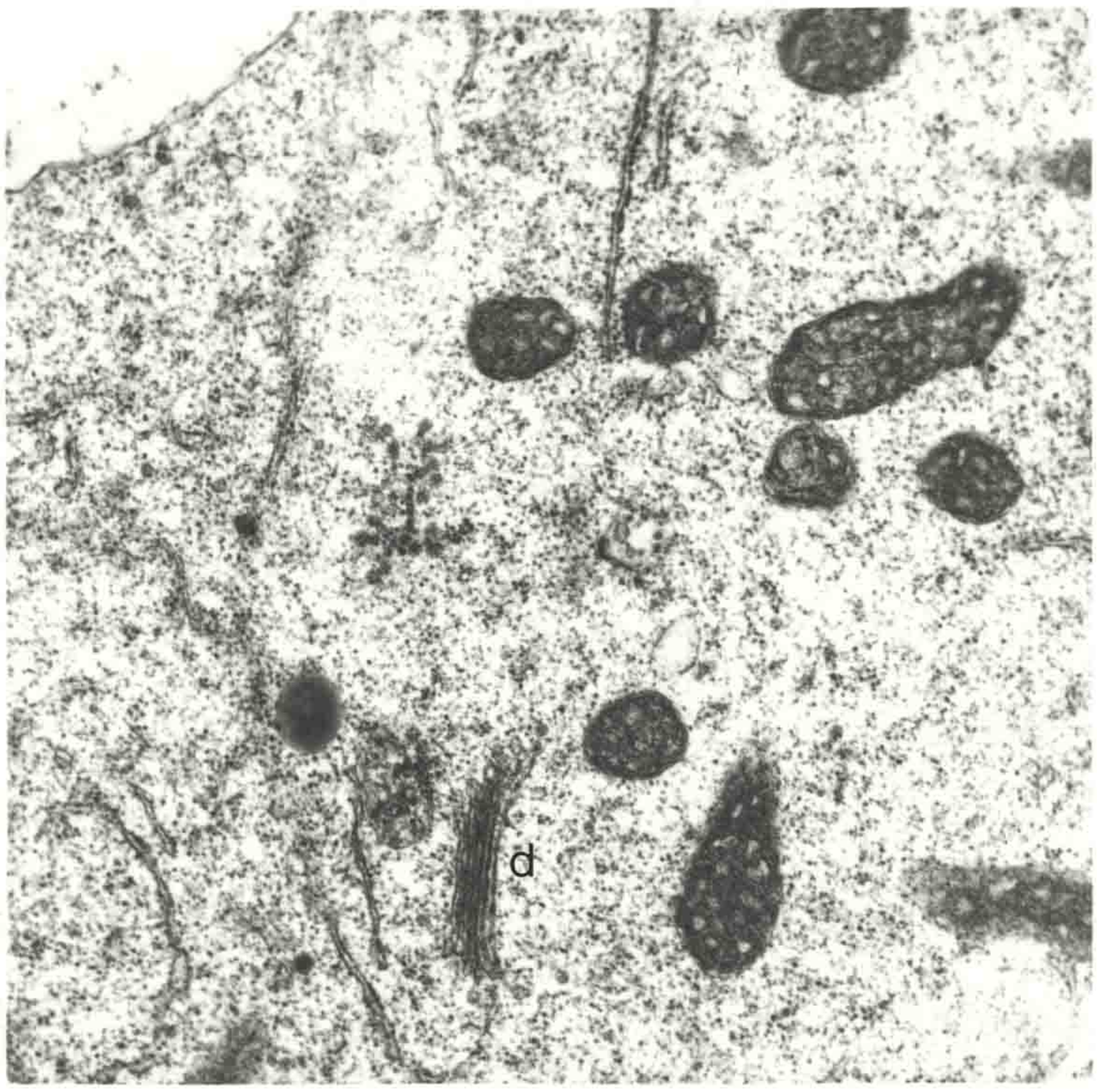


Plate 5.7

a) Electron micrograph of established callus cell with large dictyosome (d) and associated vesicles. The dense appearance of the cytoplasm is due to the abundance of ribosomes. (x12,000).

b) Section of newly-initiated callus tissue showing the same features as the established callus cells (Plate 5.5b) with a few exceptions. In this case, the cell contains fewer, larger vacuoles, fewer mitochondria of regular shape but more lipid bodies (l). The nucleolus is more diffuse in appearance and the two nucleolar regions are not clearly distinguishable. (x3,000).

a



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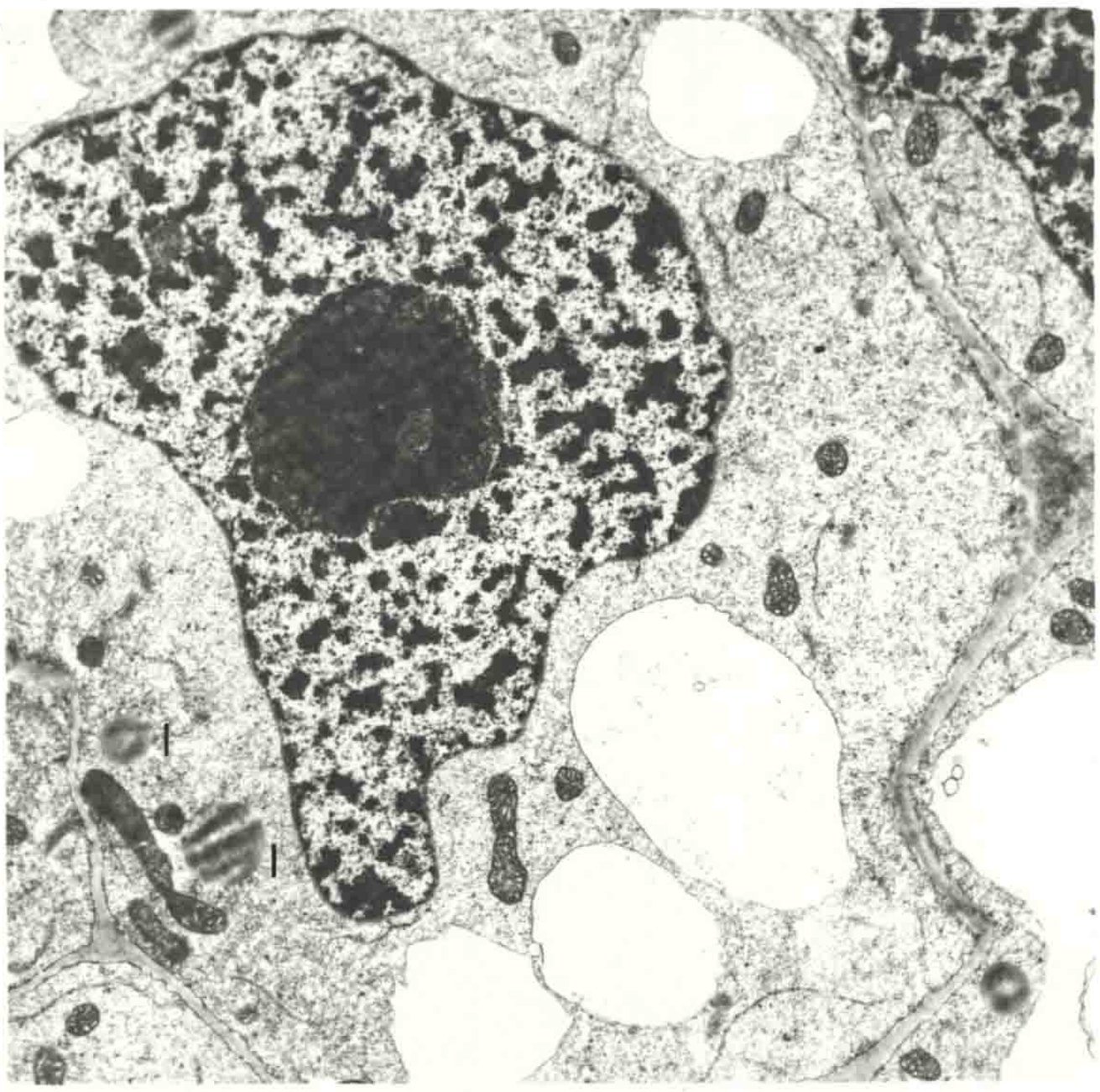


ribosomes and endoplasmic reticulum (Plate 5.7a). Often they were associated to form rough ER or the ribosomes were present as polysomes although some smooth ER and free ribosomes were still present.

The cells of established callus and those of newly initiated tissue were basically similar with a few important differences. The newly initiated callus cells had fewer, larger vacuoles although they were much smaller than those of onion cells. Fewer mitochondria were present and they did not exhibit the wide range of morphology described above but dictyosomes were more numerous and active. In the older culture cell, the nucleolus had clearly distinguishable granular and fibrous regions (Plate 5.5b) whilst those regions were intermingled in the newly initiated cell to give a more diffuse appearance to the organelle (Plate 5.7b). No thick walled cells were seen in the newly initiated callus and few plasmodesmata were evident. Lipid bodies were numerous in the newer tissue (Plate 5.8a) yet only found occasionally in established callus cells.

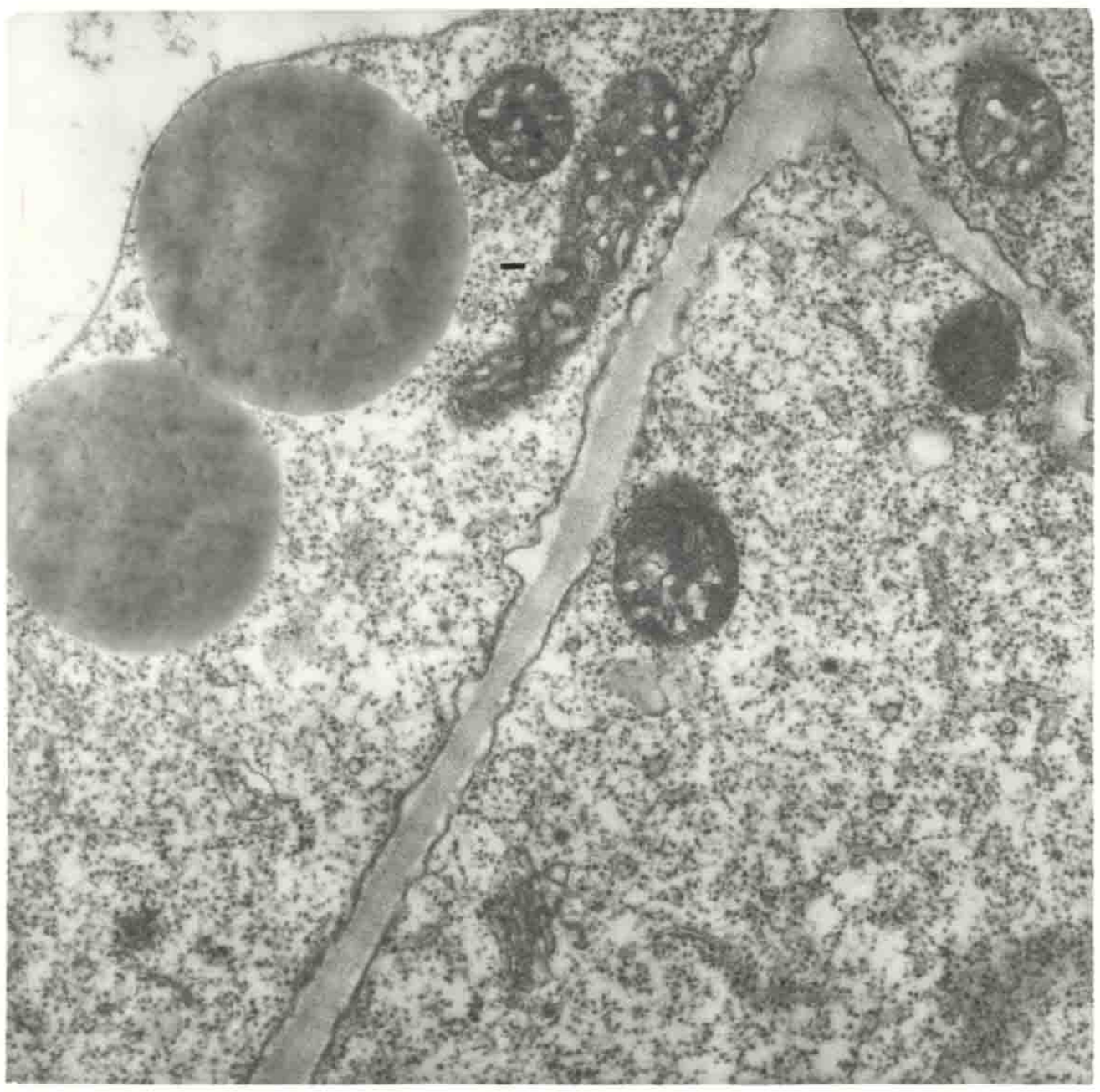
Neither type of callus appeared to possess the first type of vesicle described for onion cells. The older callus cell had a few single membrane bound vesicles which contained a small amount of loose fibrillar material (Plate 5.8b). Occasionally a vesicle was seen adjoining the cell wall (Plate 5.9a) but it is uncertain whether the contents were being discharged into the wall or taken into the cell. Similar situations were observed in newly initiated callus cells where vesicles were found fused with the plasma lemma (Plate 5.9b) and apparently transferring material between the cell wall and protoplast. Multi-vesicular bodies were also present in newly initiated callus cells (Plate 5.10a) although in contrast to those of the onion cell they were bound by a single

Plate 5.8

a) Section of newly initiated callus cells with large lipid bodies. (x12,000).

b) Section of established callus cell with single-membrane bound vesicles containing a loose, fibrillar type of material. (x8,000).

a



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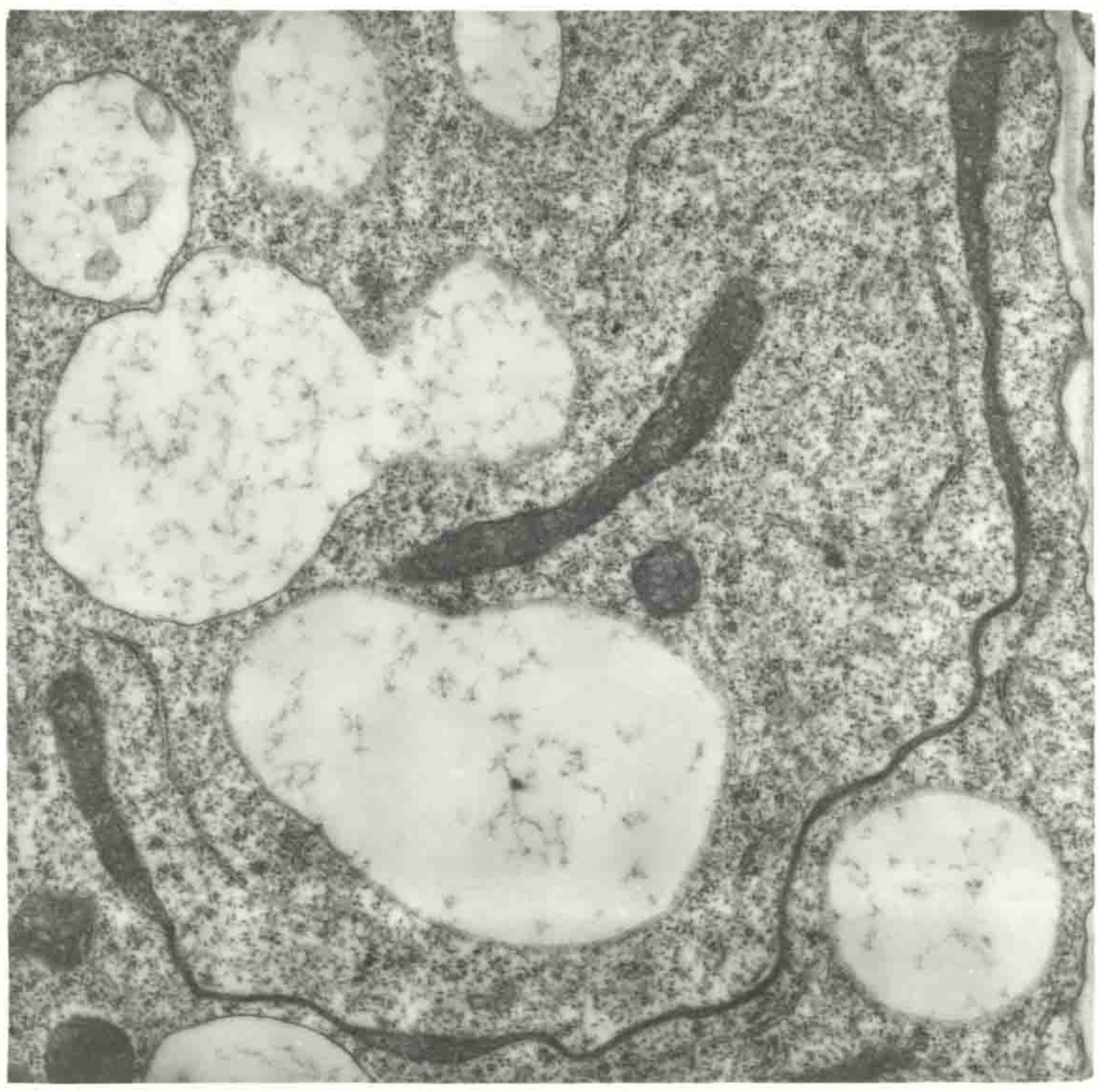
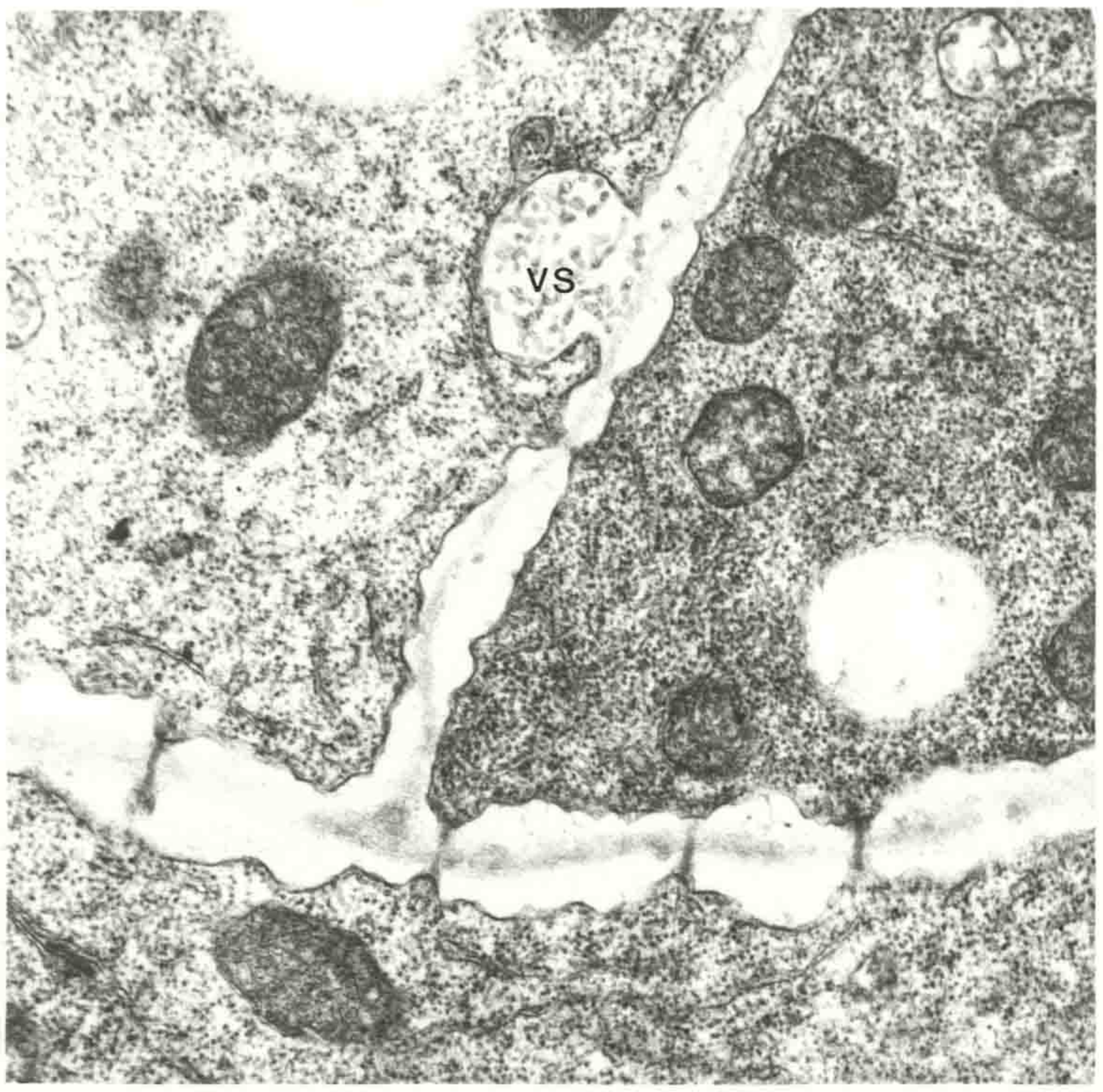


Plate 5.9

a) Electron micrograph of established callus cells containing a single-membrane bound vesicle (vs) fused with the plasma membrane. (x12,000).

b) Section of newly initiated callus cells with several empty vesicles, two of which are fused to the plasma membrane. Some of these vesicles appear to contain some cell wall material. (x20,000).

a



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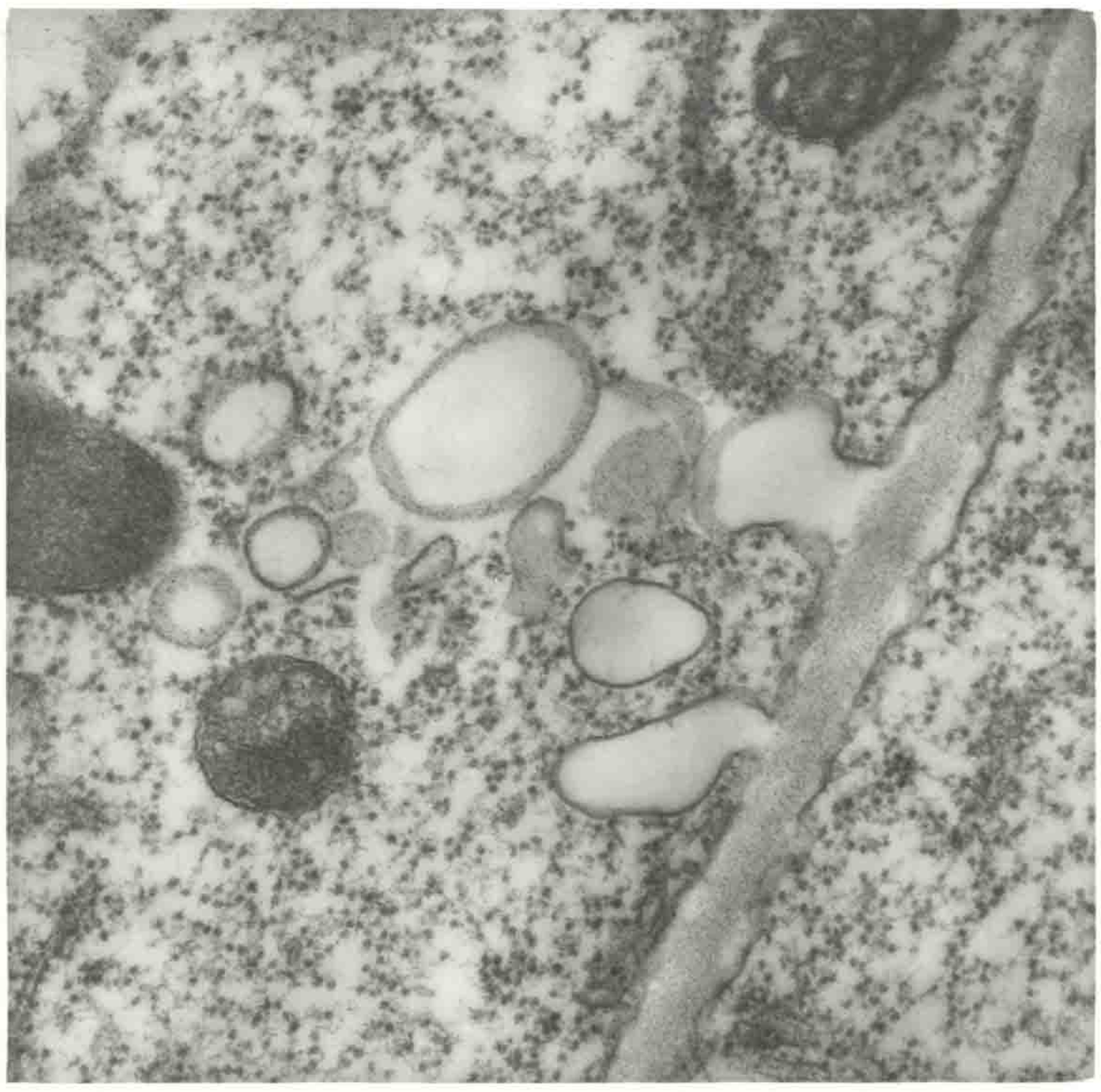
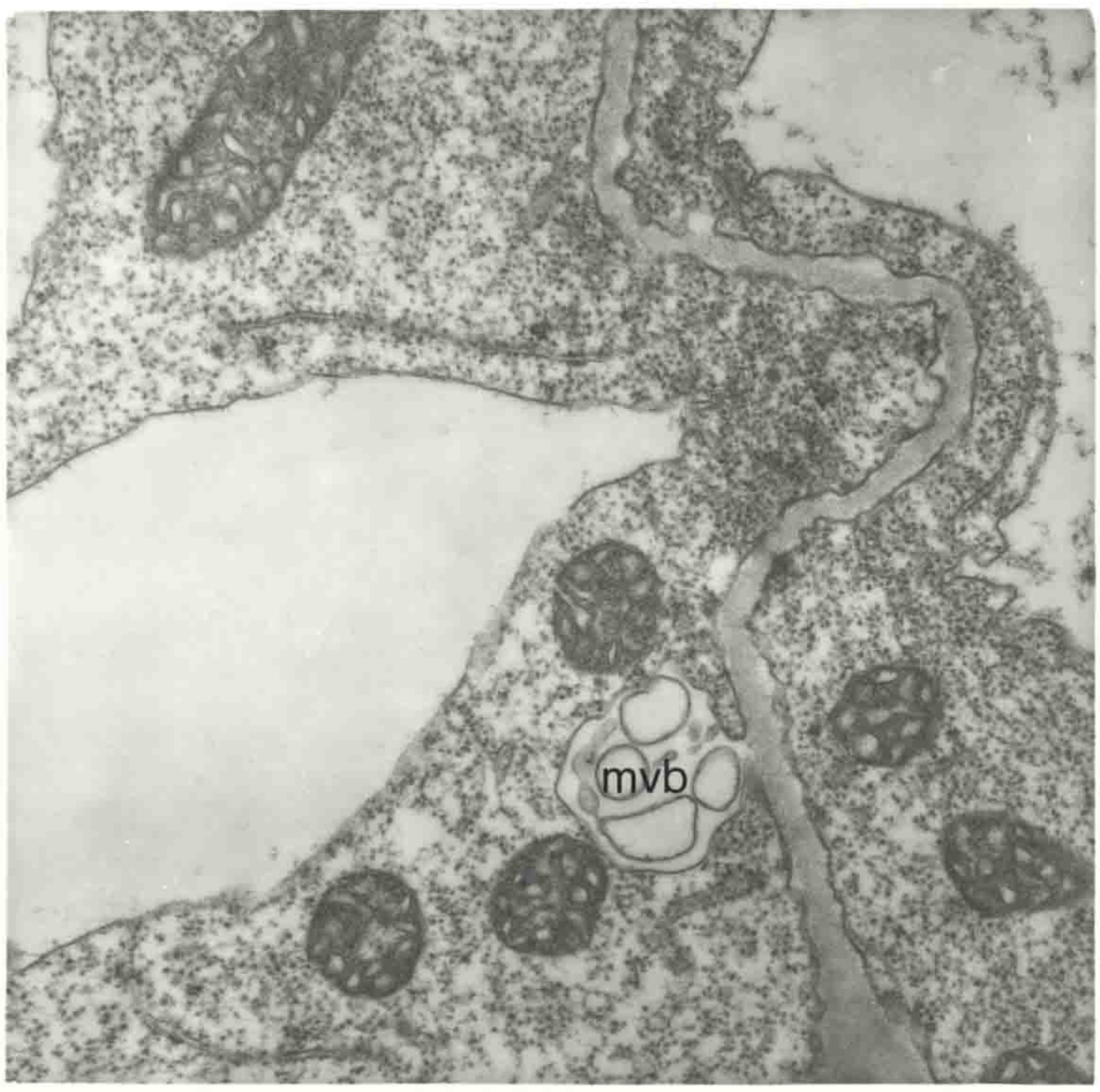


Plate 5.10

a) Section of newly initiated callus tissue showing a multi-vesicular body (mvb) in fusion with the plasma membrane. (x12,000).

b) Electron micrograph of established callus cell containing a bundle of membranes or filaments with much rough ER distributed throughout the cytoplasm. (x12,000).

a



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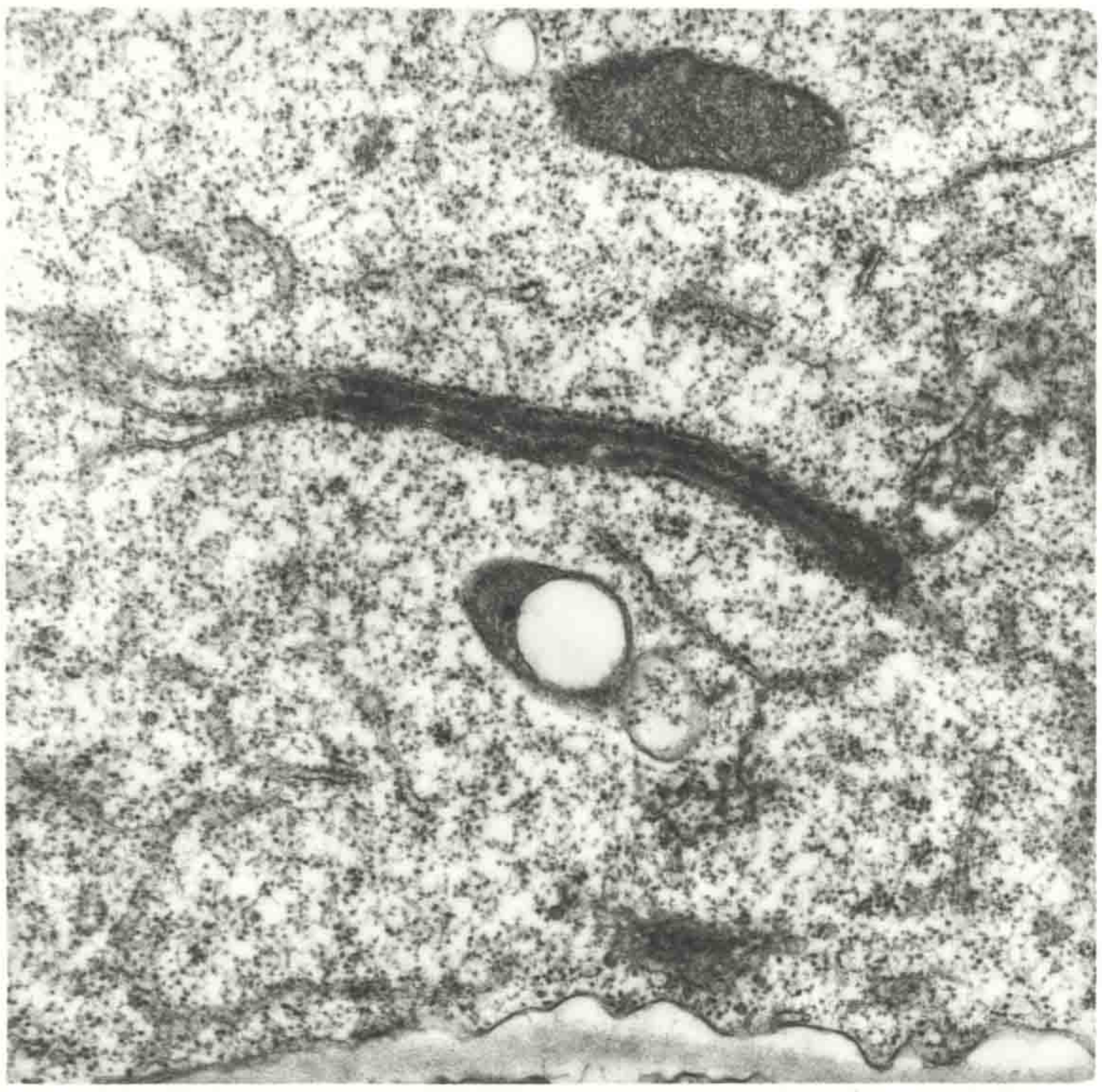


membrane. Occasionally, in cells from the older callus, groups of filaments or membrane bundles were observed (Plate 5.10b).

5.4 Discussion

The differences found between the cells of onion and callus tissues were to be expected since the intact onion tissue was from the storage part of the bulb and was therefore quiescent while the callus cells were from actively growing meristematic tissue. Some support is provided for this distinction. Yeoman (1970) found that large numbers and elongated shapes of the mitochondria in callus cells indicated an active metabolism and similarly, high metabolic activity was correlated with large numbers of mitochondria in Jerusalem artichoke tissue (Bagshaw 1969). In carrot cultures, active cells had irregularly shaped mitochondria, as observed in onion callus cells, rather than the rounded forms of the resting cell (Wilson et al. 1974). The dumb-bell shaped mitochondria in onion callus cells are thought to arise from mitochondrial division, a fact which was also noted by Sutton-Jones and Street (1968) in sycamore cell cultures. The cup-shaped mitochondria have also been detected in intact plant tissue e.g. Pteridium (Bell and Muhlethaler 1964) and Anthoceros (Manton 1961) so they are not only a feature of cultured cells. The complex mitochondria were found to be more abundant in established callus, a fact which may be a consequence of long term subculturing.

Another indication of enhanced metabolic activity in callus cultures was the large number of dictyosomes which were often found in groups and associated with vesicle formation at the edges of the stack of cisternae. The dictyosomes of quiescent cells are sometimes composed of curved cisternae with few vesicles (Mollenhauer and Morre 1966). In the onion callus cells, the dictyosomes were usually straight and associated with several vesicles. Similar observations were made by Sutton-Jones and

Street (1968) and Yeoman and Street (1973) for cells in culture. The increase in endoplasmic reticulum and particularly in rough ER and polyribosome formation in callus cells indicates that these cells are active in protein synthesis (Halperin and Jensen 1967).

The large, central, densely staining nucleus and prominent nucleolus of the callus cell also signifies high metabolic activity in contrast with the flattened nucleus of the onion cell. The lobed appearance of the nuclei was also reported by Vasil (1970) for sweet pea callus cells where it was suggested that invagination of the nuclear membrane produced a greatly increased surface area for transport of materials from the nucleus to the cytoplasm. In a dividing cell the nucleolus is less compact and the fibrillar and granular regions are intermingled (Hyde 1967) as against the nucleolus of a resting cell in which the two regions are clearly distinguishable. The uniform state of the nucleolus was seen in newly initiated callus cells only, while in the established callus cell the nucleolus was more characteristic of a quiescent cell with two distinct regions. This would suggest that the newly initiated callus cells were more actively dividing than the cells of the older callus. In fact, this supposition is supported by observations of the growth of newly initiated callus which grew at a faster rate than the well established callus clone.

Plasmodesmata were found only in callus cells. They have also been reported in cultured cells of tobacco and oat root tissue (Withers and Cocking 1972), cells of Ammi visnagi (Fowke et al. 1973) and in crown gall callus of tobacco (Spencer and Kimmins 1969). Thickenings of the cell wall in onion callus cultures were described by Davey et al. (1974) and in Acer cells

by Davey and Street (1971) but these structures were absent from the onion callus cells examined here.

In spite of the fact that callus cells are obviously more metabolically active than bulb cells, they are still incapable of synthesising the full complement of flavour compounds. This suggests that the callus cells may lack some structural feature associated with differentiation that enables the synthesis and accumulation of flavour compounds to occur. The most obvious qualitative difference between onion and callus cells was the type of vesicle present. Multi-vesicular bodies as found in onion cells have been reported by Wilson et al. (1974) and Halperin and Jensen (1967) for cultured carrot cells. They also describe inclusions in the cytoplasm of whorled sheets of membrane or membranes in the form of tight bundles of fibrils. The former were found in onion cells and bundles of fibrils in callus cells. This is in agreement with Wilson et al. (1974) where it was reported that bundles of fibrils are associated with active metabolism. Mahlberg et al. (1974) describes in callus cells of Helianthus annus L. and Glycine max, the presence of secondary vesicles and complex accumulations of membranes consisting of spherical, tubular and laminar structures in the cytoplasm. In the carrot cell cultures of Halperin and Jensen (1967) the single membrane bound vesicle contained several similar vesicles whilst in the onion cell the vesicles appeared to be bound by double membranes. In the carrot cells, multi-vesicular bodies were formed by pinching off of vesicles from the dictyosome cisternae, which then fused with the plasma lemma and deposited their contents into the wall spaces. This would indicate an involvement in cell wall synthesis which is

consistent with the dividing cells of callus tissue.

The vesicles most characteristic of onion cells were those adjoining the cytoplasm and filled with material of unknown composition, which were not observed in callus tissue. These vesicles may be storage sites for food reserves or for the large amounts of flavour compounds present in the onion tissues. Since the lachrymatory factor is only released when onion cells are damaged, Becker and Schuphan (1975) suggested that the flavour compounds and the alliinase enzyme which degrades them must be separated in some way. Since production of the lachrymatory factor is almost instantaneous, it is likely that the two factors are separated spatially rather than temporally. Thus, it follows that the enzyme and substrate will only come into contact when the cell is damaged or begins to senesce with accompanying membrane breakdown. Becker and Schuphan (1975) suggested that either the substrate (alkyl cysteine sulphoxide) is stored in the vacuole or the enzyme is stored in special lysosomes. It is reasonable to suppose that the substrate could also be stored in special vesicles such as those described for onion cells. Such vesicles were absent from callus cells although some vesicles in the cytoplasm were found to contain a small amount of material of similar composition to that stored in the larger vesicles of onion cells. It is known that callus cells contain a low level of flavour compounds compared with the amount stored in onion tissue. It is possible that the apparently specialised vesicles of onion cells are only formed when the cell contains high levels of storage compounds. Since larger amounts of flavour compounds are present in differentiated callus tissue it would be interesting to examine cells from such tissues to determine whether any

specialised vesicles are formed during the differentiation process.

CHAPTER 6

SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITY

6.1 Introduction

The production of the characteristic odour and lachrymatory effect which results when onion tissue is cut or damaged is well known. It has been suggested that the rapid formation of the onion flavour volatiles only occurs on cell damage because in the intact cell the flavour precursor compounds and the degradative enzyme alliinase are separated in some way, probably spatially (Virtanen 1962, 1965, Granroth 1970, Schwimmer and Friedman 1972, Schutte 1974). Many secondary products are accumulated or stored in either the central vacuole or in special storage vesicles in the cell. In the case of the onion, it is likely that the flavour precursor compounds are stored in the specialised vesicles described in Chapter 5, whilst the alliinase enzyme is stored in special lysosomes or occurs freely in the cytoplasm or central vacuole. Conversely, it was suggested by Becker and Schuphan (1975) that both enzyme and substrate may occur simultaneously in the cell but only react together when the enzyme is activated by oxygen on cell damage. However, the almost instantaneous production of onion flavour when the tissue is crushed does not support this view (MacLeod 1970). The alliinase enzyme was reported to be a general property of onion cells (Becker and Schuphan 1975) and Freeman (1975) showed that whilst the levels of flavour precursors were highest in the stem and inner tissues of the bulb, these compounds were present in all parts of the plant. Thus, it is likely that spatial separation of the two reactants will involve compartmentalisation within the cell rather than the storage of the flavour precursors in a

particular organ of the plant.

The localisation of enzymes in particular cell organelles is well known and is a useful criterion in the characterization of subcellular fractions. For example, cytochrome oxidase and succinic oxidase are known as marker enzymes for mitochondria since they are exclusively found in these organelles, whilst chlorophyll and nitrite reductase are reference compounds for the chloroplast (Hallaway 1968).

The technique of cell fractionation by differential centrifugation is a method widely used in the study of enzyme distribution within the cell (Sanwal 1963, Roodyn 1965, Novikoff and Holtzman 1970, Miflin and Beevers 1974). This method separates the structural elements of the cell by means of the differences in their sedimentation properties, then the resulting fractions can be characterised by light and electron microscopy, histochemistry and assays for enzymes and other reference compounds such as DNA, protein and chlorophyll.

Although much work has been carried out on the extraction, purification and characterization of alliinase enzymes from onion (Kupiecki and Virtanen 1960, Schwimmer et al. 1960, 1964, Schwimmer and Mazelis 1963), garlic (Stoll and Seebeck 1951, Mazelis and Crews 1968) and members of the Cruciferae (Mazelis 1963), little has been done to locate the enzyme in the cell. Schwimmer and Mazelis (1963) reported the presence of alliinase activity in the 11,000 x g sediment recovered from an homogenate of etiolated onion shoots. This activity had not sedimented at

5,000 x g and no activity was found in the supernatent resulting from centrifugation at 11,000 x g. However, these fractions were not characterized and attempts to prepare similar fractions from mature onion bulbs were unsuccessful.

Later work by Schwimmer (1969) with onion bulb preparations reported that one half of the enzyme activity in the original homogenate was present in the pellet obtained by centrifugation for thirty minutes at 34,000 x g. Experiments showed that the activity was heterogeneously distributed throughout the cellular components of the particulate fraction. It was suggested that alliinase activity may be due to two or more enzymes in the particulate fraction of onion homogenates. Evidence for at least two alliinase enzymes was presented by Spare and Virtanen (1963). However, no further experiments were reported and so the intracellular location of alliinase still requires clarification.

Similarly, few investigations have been carried out on the site of synthesis in the cell of the flavour precursor compounds, the S-alkyl and S-alkenyl cysteine sulphoxides. As a result of a series of sensory, histochemical and autoradiographic tests, Becker and Schuphan (1975) concluded that the substrates of alliinase were stored in the bundle sheath cells of the vascular tissue, but no intracellular location was suggested.

Granroth (1970) unsuccessfully attempted to carry out the conversion of CPC to Pren Cy SO in cell free homogenates of Allium tissue. However, no experimental details were provided. The conversion of S-propyl - L - cysteine to its sulphoxide was

reported to occur in a microsomal preparation from rat liver tissue (Ebbon and Callaghan 1968). Since this reaction was shown to be characteristic of one particular fraction of a cell free homogenate, and the oxidation of thioethers to their corresponding sulphoxides has been observed in several biological systems, it is reasonable to suppose that the system in onion tissue will also be associated with a particular fraction of the tissue homogenate.

The work reported here was concerned with the development of a suitable fractionation method for use with onion homogenates in the preliminary investigation into the subcellular location of the alliinase enzyme and the biosynthetic pathway leading to the production of Pren Cy SO.

6.2 Materials and Methods

6.2.i Fractionation of tissue homogenates.

Onion bulbs of English origin but unknown variety were purchased from a local market. Only the inner portion of the bulb was used, after the outer papery leaves, basal disc and roots were removed. This material was more suitable for provision of a high yield of active enzyme since this part of the bulb is metabolically active, whilst the outer fleshy layers function mainly as storage tissue. Only healthy, yellow callus tissue was used in these experiments, any brown material being discarded.

The tissue was homogenised in extraction medium (2 ml g^{-1} fresh weight) consisting of 50mM Tris HCl buffer, pH 8.5 (Trizma Base : Sigma Chemical Company) containing 0.5M sucrose, 1mM EDTA and 0.1% v/v mercaptoethanol. The medium was semi-frozen before use and the mercaptoethanol added immediately before homogenising. All operations were carried out at $4^{o}C$ and all glassware and materials were chilled overnight before use. Homogenisation was carried out for two five second periods at high speed in an MSE Ato-mix blender. The resulting suspension was filtered through four layers then eight layers of muslin and the filtered homogenate fractionated by differential centrifugation. The pH of the prepared homogenate was 8.3.

A known volume of homogenate was centrifuged for 10 minutes at 2,000 g in an MSE Superspeed 65 centrifuge. The pellet was

reserved and the supernatent centrifuged for 10 minutes at 10,000 g. This process was repeated with centrifugations at 20,000 g (10 min.), 50,000 g (10 min.), 60,000 g (17 min.), and 100,000 g (50 min.). Each pellet was resuspended in a known volume of 0.1M sodium pyrophosphate buffer, pH 9.0, using a Potter-Elvehjem type homogeniser and the volume of the final supernatent was recorded. Samples of homogenate and final supernatent were reserved and all samples stored at -20°C.

The assay for alliinase activity was carried out as described in Section 4.2 vi using synthetic propyl cysteine sulphoxide as a substrate and soluble protein was estimated as described in Section 4.2 iv following the method of Lowry et al. (1951).

6.2.ii Assay for synthesis of Pren Cy SO from CPC.

This assay was designed to test fractions of cell free homogenates for the presence of the biosynthetic pathway which converts CPC to Pren Cy SO in intact onion tissues.

The reaction mixture contained 0.4 ml 0.1M sodium pyrophosphate buffer, pH 9.0, 0.1 ml 0.5mM pyridoxal phosphate, 0.3 ml 50mM CPC, 0.1 ml freshly prepared alliinase solution at a concentration of 1 mg protein ml^{-1} and 0.1 ml sample. As in the assay for alliinase activity, 0.1 ml of the reaction mixture was removed at various times up to thirty minutes and the reaction stopped by the addition of 1 ml 10% TCA. Samples were then assayed for pyruvate following the method of Schwimmer and Guadagni (1962) as described in Section 4.2 iii.

6.2 iii Preparation of alliinase solution

Alliinase was extracted from mature onion bulbs as described in Section 4.2 v and freeze dried before storage at -20°C. Solutions were always freshly prepared before use by dissolving in 0.1M sodium pyrophosphate buffer, pH 9.0, at a concentration of 1 mg protein ml^{-1}. The solution retained its enzymic activity with only slight decline for at least three months when stored at -20°C, but activity was rapidly lost in a few days if the enzyme solution was stored at 4°C.

6.3 Results

The method of fractionation and differential centrifugation described was the final method adopted after several modifications had been tested. Tissue was originally ground up in a mortar and pestle but since it was not possible to standardise this method it was replaced by the use of an electric blender. This was more suitable for these experiments where a high yield of enzyme was not required, but where the extraction procedure needed to be done as rapidly as possible to reduce the possible loss of enzyme activity from its particular location in the cell.

Investigation of the pH changes occurring during homogenisation showed that the pH of the buffer was reduced from 7.5 to 6.5 due to the high organic acid content of the onion tissue. Thus, a pH of 8.5 was used, since, on homogenising, only a slight change to 8.3 resulted. This pH was more suitable for extraction of alliinase since the enzyme is reported by Schwimmer and Mazelis (1963) to have pH optima of 7.5, 8.3 and 8.8 in phosphate, Tris and pyrophosphate buffers respectively. Optimum buffering capacity was obtained by using Tris HCl buffer, pH 8.5 at a concentration of 50mM. Other buffers tested included Honda medium (Honda et al. 1966) but this resulted in only 50% recovery of alliinase activity whilst by using Tris buffer, 90% of the activity present in the homogenate was recovered in the combined pellets and supernatent after differential centrifugation.

In order to use the production of pyruvate as a direct measure of the alliinase activity present in each fraction it was necessary to ascertain that pyruvate production varied lineally with enzyme concentration and reaction times. Fig. 6.1 shows that linear graphs were obtained when the volume of alliinase in 1 ml reaction mixture was varied between 10 and 100 µl and the reaction times varied between 0 and 20 minutes. After 20 minutes, the pyruvate production declined but for most purposes a reaction time of 5 minutes was adequate to provide a measurable amount of pyruvate. As a source of alliinase enzyme, volumes of the final supernatent from the differential centrifugation procedure were used. However, similar results were obtained when solutions of freeze dried alliinase preparations were tested.

Each component of the reaction mixture used in the assay for alliinase activity, sodium pyrophosphate buffer, pydidoxal phosphate, propyl cysteine sulphoxide and the alliinase-containing sample, was tested for its contribution to the colour reaction which occurs when 2,4-dinitrophenylhydrazine is added to the sample. The experiment confirmed that, under the specified conditions of the reaction, there was no spontaneous breakdown of substrate which, in this case, was synthetic propyl cysteine sulphoxide. It also confirmed that any increase in optical density recorded with an increase in time, was due to enzymically produced pyruvate which results when alliinase degrades the substrate.

Fig. 6.1 Effect of varying volumes of alliinase preparation on the amount of enzymically-produced pyruvate.

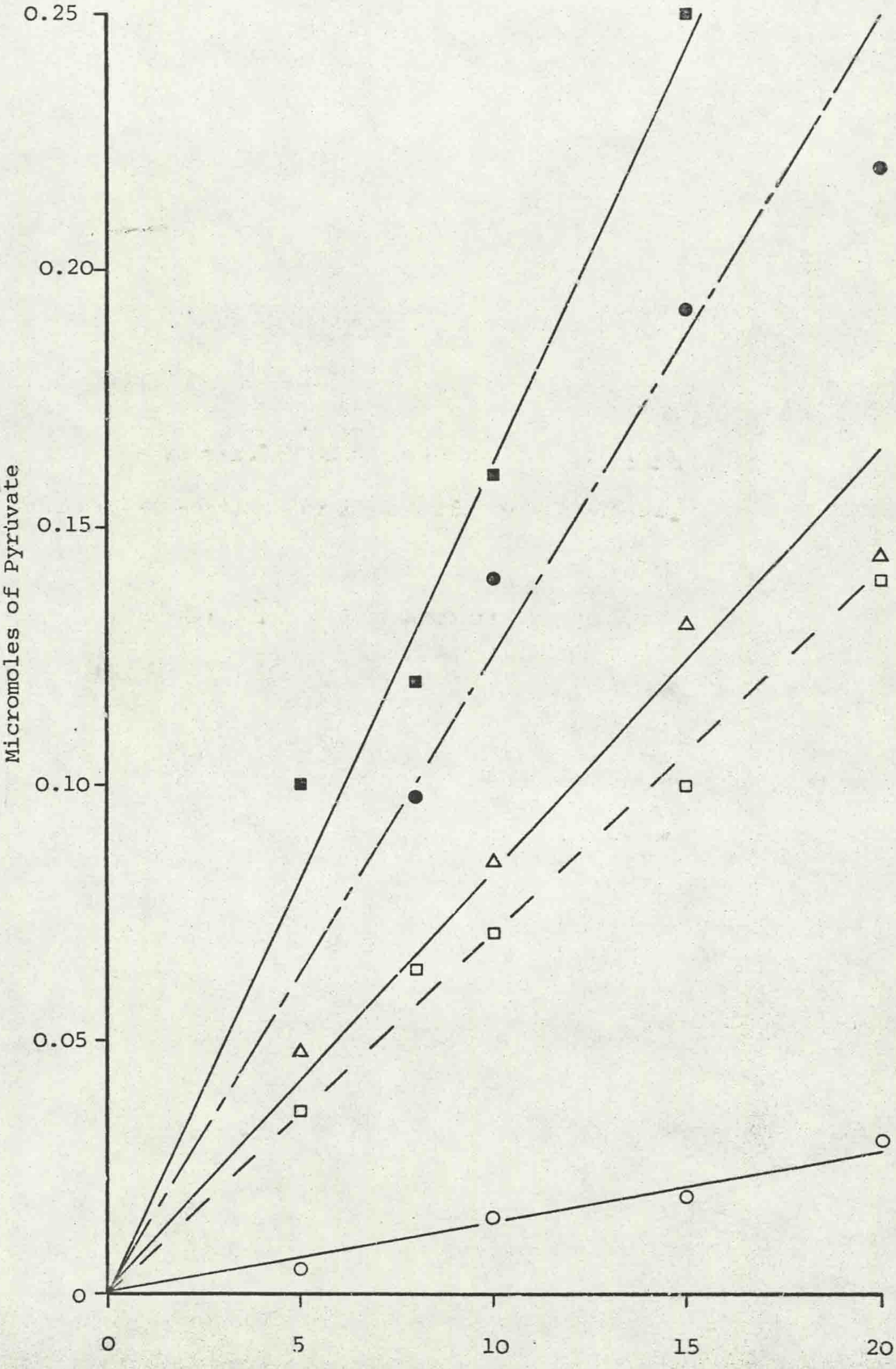
o 10 µl alliinase solution ml^{-1} reaction mixture

□ 30 µl

△ 50 µl

● 80 µl

■ 100 µl


0.25
0.20
0.15
0.10
0.05
0
Micromoles of Pyruvate
0
5
10
15
20
Minutes

Several fractionations of onion bulb tissue were carried out and the results of three of these are presented here. Table 6.1 shows the total units of enzyme activity recoverable in each fraction, where one unit of enzyme activity is that which produces 1 µmole pyruvate in five minutes at 25°C under the specified conditions of the reaction mixture. Approximately 90% of the total activity present in the crude homogenate was recoverable in the resulting fractions, indicating that the conditions of the procedure were suitable for alliinase extraction and assay. The variations in the levels of total activity in the homogenate may be due to differences in storage conditions, duration of storage or seasonal differences in the fresh material.

Only 4% of the total recoverable activity was sedimentable (Table 6.1), the remainder being present in the soluble fraction. Only 1.5% of the recoverable activity was present in pellet 6.

Occasionally, the homogenate was subjected to the final spin only and again it was found that only 10% of the activity was lost and that 5% of the recoverable activity was sedimented. Therefore, it was assumed that no enzyme activator or inhibitor was present which would normally be separated from the enzyme as a result of the purification which occurs during differential centrifugation.

From the result of sample 1 (Table 6.2) where pellet 6 had a high specific activity it was thought that much of the enzyme activity was associated with this pellet and that the activity in the soluble fraction was possibly due to loss of the loosely-bound enzyme from the membrane fraction into the supernatent. However,

Table 6.1 Total units of alliinase activity in each fraction of onion bulb and callus tissue homogenates.

Fraction	Units of alliinase activity					
	Onion bulb samples			Callus samples		
	1	2	3	1	2	3
Homogenate	438.30	338.70	692.10	128.10	128.10	91.20
Pellet 1	0.57	0.00	3.42	8.25	2.42	2.13
Pellet 2	2.13	1.56	6.64	5.55	2.70	4.56
Pellet 3	0.00	0.99	3.60	3.99	3.84	3.71
Pellet 4	2.99	1.85	2.66	2.85	3.42	3.27
Pellet 5	0.00	2.28	2.46	2.70	2.00	2.99
Pellet 6	11.96	2.70	6.26	3.84	3.84	4.70
Supernatent	384.21	284.31	605.64	109.60	65.46	50.35
Total units in pellets + supernatent	401.85	293.69	630.68	136.78	83.68	71.71
% activity lost	8.32	13.29	8.87		34.68	21.37
% activity sedimentable	4.39	3.19	3.97	19.87	21.77	29.79
% activity in pellet 6	2.98	0.92	0.99	2.81	4.59	6.55

(1 unit of alliinase activity is that which produces 1μ mole of pyruvate in five minutes at 25°C under the specified conditions of the reaction mixture).

Table 6.2 Specific activity of the alliinase enzyme in each fraction of onion bulb and callus tissue homogenates.

Fraction	Specific activity of alliinase enzyme (μ moles pyruvate mg^{-1} protein)					
	Onion bulb samples			Callus samples		
	1	2	3	1	2	3
Homogenate	9.50	7.24	5.74	4.38	3.43	2.41
Pellet 1	1.60	0.00	1.99	4.27	1.42	1.11
Pellet 2	1.09	1.24	1.87	1.70	0.88	1.31
Pellet 3	0.00	0.57	1.40	2.80	1.88	1.67
Pellet 4	3.69	3.32	1.46	3.45	2.53	2.18
Pellet 5	0.00	5.63	1.78	5.55	1.93	2.46
Pellet 6	38.88	6.67	2.55	3.20	2.31	2.59
Supernatent	12.56	7.21	5.26	7.11	2.80	1.82

further samples failed to support this suggestion and it is now believed that the alliinase enzyme is soluble and not associated with a particulate fraction of the cell. Activity in the pellets is most likely to be due to contamination by small volumes of supernatent left in the pellet at each stage. However, on one occasion when the pellets were washed before being resuspended in buffer and assayed the activities in each fraction were identical to the activities found in unwashed pellets.

The crude homogenate was assayed in the presence and absence of Triton X 100. This would have the effect of solubilising the membranes, so releasing membrane-bound enzymes which would then have a higher activity in the detergent treated sample than in the untreated sample. However, the results of the two assays were identical, indicating that alliinase is not associated with the membranes. Further confirmation is required from treatment of each fraction, but this was not tested.

Similar fractions were prepared from sprouting bulb tissue and green leaf tissue but in each case the results paralleled those described above with most activity found in the soluble fraction.

Due to limited availability of callus material, only three fractionations were possible. Table 6.1 shows the total units of activity present in each fraction. In all cases, results of the callus fraction assays are directly comparable with those of the onion fractions. In the callus fractionation, approximately 28%

of the total activity in the crude homogenate was lost during the centrifugation process. Table 6.1 indicates that about 23% of the recoverable activity was sedimentable with approximately 4.5% activity in pellet 6. However, many more fractionation results are required before any definite conclusions can be drawn. Since 77% of the alliinase activity was found in the soluble fraction, it was assumed that, as in the onion, the enzyme is soluble and not normally associated in the cell with any particular membranes or organelles. Supporting this hypothesis is the fact that the total units of enzyme activity for each pellet (onion and callus) are all very similar in magnitude and presumably result from contamination.

The specific activities of the callus fractions are shown in Table 6.2 and, as found for onion fractions, no fraction was consistently found to have a significantly higher specific activity than the other fractions.

In the assay for the operation of the biosynthetic pathway, only freshly prepared fractions of onion homogenates were used, but no reaction was observed. Homogenates from sprouted bulb material and green leaf material also failed to produce any reaction. In one experiment, three series of pellets were prepared and then the three pellets produced at each centrifugation were combined and resuspended in 2 ml sodium pyrophosphate buffer instead of the usual volume of 4.5 ml. However, these concentrated solutions also failed to show evidence of biosynthetic capacity. Similarly, no reactions were detected when bulb tissue was homogenised in Tris

buffer at four different pH values of 5.5, 6.5, 7.5 and 8.5, and there was no noticeable difference in the activities of the four samples. Finally, fractions of an onion bulb homogenate were tested for the conversion of propyl cysteine to its sulphoxide but this pathway was not found to be operational in any fraction. Callus fractions were not tested for any biosynthetic capacity.

6.4 Discussion

The results reported here indicate that alliinase is a soluble enzyme which, in the intact cell, is probably located in the central vacuole or in special lysosomes. The central vacuole and lysosomes would be ruptured during the homogenisation procedure since a much more gentle method is required to extract these organelles in an undamaged condition. Thus, the contents of the central vacuole and lysosomes would be released into the soluble fraction. It is also possible that the enzyme is present in the general cytoplasm and is not associated with any structural feature of the cell. It is believed that in the case of the onion cell, the specialised vesicles which were present in bulb tissue but absent from callus tissue, as reported in Chapter 5, may be the sites of accumulation of the flavour precursor compounds in onion cells. These compounds may also be synthesised in these vesicles, or the vesicles may be simply storage sites. On the assumption that the flavour precursor compounds are located in storage vesicles in the intact cell, the failure of the biosynthetic pathway to operate in cell free systems may have been due to the loss of necessary cofactors or energy producing systems when these vesicles were ruptured during homogenisation. For example, when Ebbon and Callaghan (1968) demonstrated the conversion of propyl cysteine to its sulphoxide in the microsomal fraction of rat liver homogenates, an NADPH generating system was

included in the reaction mixture. Such a system may also be necessary in the case of onion tissue homogenates to enable the biosynthetic pathway to operate. Alternatively, the pH of the reaction mixture may have been unsuitable for measurable activity of the pathway enzymes to occur, since the conditions of the reaction mixture were originally designed to test for alliinase activity.

Further work is required on the characterisation of the fractions obtained. This may be done with the use of assays for marker enzymes such as isocitrate lyase in glyoxysomes (Pitt and Galpin 1973) and also reference compounds such as nucleic acids. Thus, it may be possible to assign alliinase activity to a particular fraction if it can be shown that alliinase activity always occurs in a constant ratio with another enzyme or reference compound. For example, the constant ratio of nitrite reductase to chlorophyll shows that this enzyme is associated with the chloroplast.

The series of centrifugations used in the method described provide a series of g x minute values for the sedimentation of each subcellular fraction. Thus, a sedimentation curve could be constructed for several enzyme activities. This would show in which fraction each major organelle was present and also the degree of contamination by the components of that organelle in other fractions.

Similarly, examination of the fractions by light and electron microsopy will provide information concerning the major component of each fraction, the degree of integrity of the

organelles and also the amount of contamination from other organelles. For instance, it is possible that a low enzyme activity could be due to the activity being present in trace amounts in the major organelle of the fraction, or in larger amounts in a contaminating organelle. Microscopic examination of the fractions would clarify such situations. Similarly, if much organelle damage has occurred during the isolation procedure, then many fractions will be contaminated by enzyme activities from fragments of other organelles.

It is obvious that much more work is required on the location of the biosynthetic pathway in intact onion tissues before the methods can be extended to include callus tissues. However, the procedure described here does provide a suitable method of subcellular fractionation for the plant material in question.

CHAPTER 7

CHEMICALLY-INDUCED MUTAGENESIS IN CALLUS CULTURES

7.1 Introduction

It is now well established that callus cultures of onion produce a level of flavour precursors which is significantly lower than that found in the intact plant. Nevertheless, a wide range of variability in secondary product synthesis exists in cultured onion tissues as shown by Selby and Collin (1976). Several other examples of variability have been reported in the literature. Townsley (1977) described the selection of stable cell populations from carrot cell suspension cultures that exhibited a wide range of synthetic ability in the production of carotenoid pigments. He suggested that the secondary compound production from plant cells may be improved by the propagation of selected cell populations. Other examples of variation in the synthesis of secondary products in cultured tissues include the different amounts of phytochrome in clones of wild carrot tissue of common origin (Wetherell and Koukkari 1970), anthocyanin content of cultured cells of Phaseolus vulgaris (Nickell and Tulecke 1959) and Haplopappus gracilis (Blakely and Steward 1961) and of nicotine produced by Nicotiana rustica callus cultures (Tabata and Hiraoka 1976).

In many of these cases the observed frequency of variant clones is much greater than could be due to the natural level of mutation, therefore suggesting that this is due to epigenetic variation (Maliga 1976) which is inherent in the cultured

tissue. In other words, the variation is probably not due to permanent, hereditary changes in the DNA but simply changes in gene expression which manifest themselves as phenotypic alterations. Thus, there is much evidence in the literature to suggest that a cell culture is composed of a mixed population of cells, each of which would appear to be sufficiently stable when cultured alone to produce characteristic amounts of secondary product. Consequently, it should be possible to select and maintain high yielding clones (Townsley 1977) for the 'in vitro' production of secondary products in quantities suitable for industrial application. The industrial potential of higher plant suspension cultures in continuous culture systems have been described in several reviews (Mandels 1972, Street 1977a, 1977b, 1977c).

The main purpose of this investigation was to select variants of onion callus with the ability to produce significant levels of flavour precursor compounds. In the first instance, it was necessary to develop a suitable selection procedure which was specific for flavour precursor synthesis. The selection procedure to be used involved the growth of callus clones on agar medium containing physiologically high levels of biosynthetic pathway intermediates. Two levels of intermediate were to be selected, one of which allowed some callus growth and the other which prevented growth. This would provide a measure of the threshold level of intermediate on which callus material could survive but show no growth. Thus, any clones able to survive

and grow on the threshold media were presumed to be able to operate the biosynthetic pathway and metabolise the exogenously supplied intermediates to the end product, Pren Cy SO. The ability of the callus to operate the biosynthetic pathway was demonstrated by feeding ^{14}C- cysteine to cultured tissue (Chapter 3) but the presence of such high levels of intermediates in the medium would be expected to result in the death of all cells except those capable of operating the pathway rapidly and synthesising large amounts of flavour precursor.

Similar selection procedures to that described above have been used to isolate naturally occurring and mutagen-induced mutants or variants. Examples include the selection of auxotrophic mutants of E.coli and Salmonella typhimurium (Kaudewitz 1959), the ferns Todea barbara and Osmunda cinnamomea (Carlson 1969) and of cultured cells of Nicotiana tabacum (Carlson 1970). In the latter two cases, cells were plated onto agar-solidified minimal medium which supported the growth of the wild type cells but not the auxotrophs. Cells were then treated with 5-bromodeoxyuridine (BU d R) which is incorporated into the DNA of actively dividing cells. Subsequent exposure to light resulted in the death of those cells containing BUdR. Thus, the remaining cells were the auxotrophic mutants which could be recovered by transferring them to supplemented media. This technique for the isolation and recovery of auxotrophic mutants was first developed for use with mammalian cell cultures (Puck and Kao 1967) and later applied to cultured cells of higher plants.

For the selection of variants resistant to compounds such as streptomycin (Binding et al. 1970, Diamond and Schiff 1974), base-analogues (Widholm 1972, 1974, Lescure 1973, Marton and Maliga 1975) and sodium chloride (Dix and Street 1975, Nabors et al. 1975), cells are plated out on agar medium (Bergmann 1960) containing the compound in question. Only those cells able to grow on the medium are selected as being resistant clones (Maliga 1976).

The natural level of variability which exists in cultured tissue can be increased by treatment of the callus cells with chemical mutagens (Dulieu 1972, Maliga 1976) and the resulting mutated or variant cells isolated using selection procedures similar to those outlined above (Widholm 1977). The two chemical mutagens used in this study were nitrous acid and N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

In many cases, ionising radiations such as X-rays (Broertjes and Leffring 1972, Vardi et al. 1975, Broertjes et al. 1976) and γ-rays (Furuya et al. 1970) have been used to induce mutations in whole plants, cultured higher plant cells and protoplasts. However, these are only considered as a last resort if all other mutagen treatments fail, since these radiation treatments often result in chromosomal breakage, thus leading to structural changes which would complicate genetic analysis of the mutants. Consequently, chemical mutagens are more widely used because they have specific effects on the DNA of the cell. Some mutagenic agents such as 5-bromouracil (5-BU) are known as base analogues

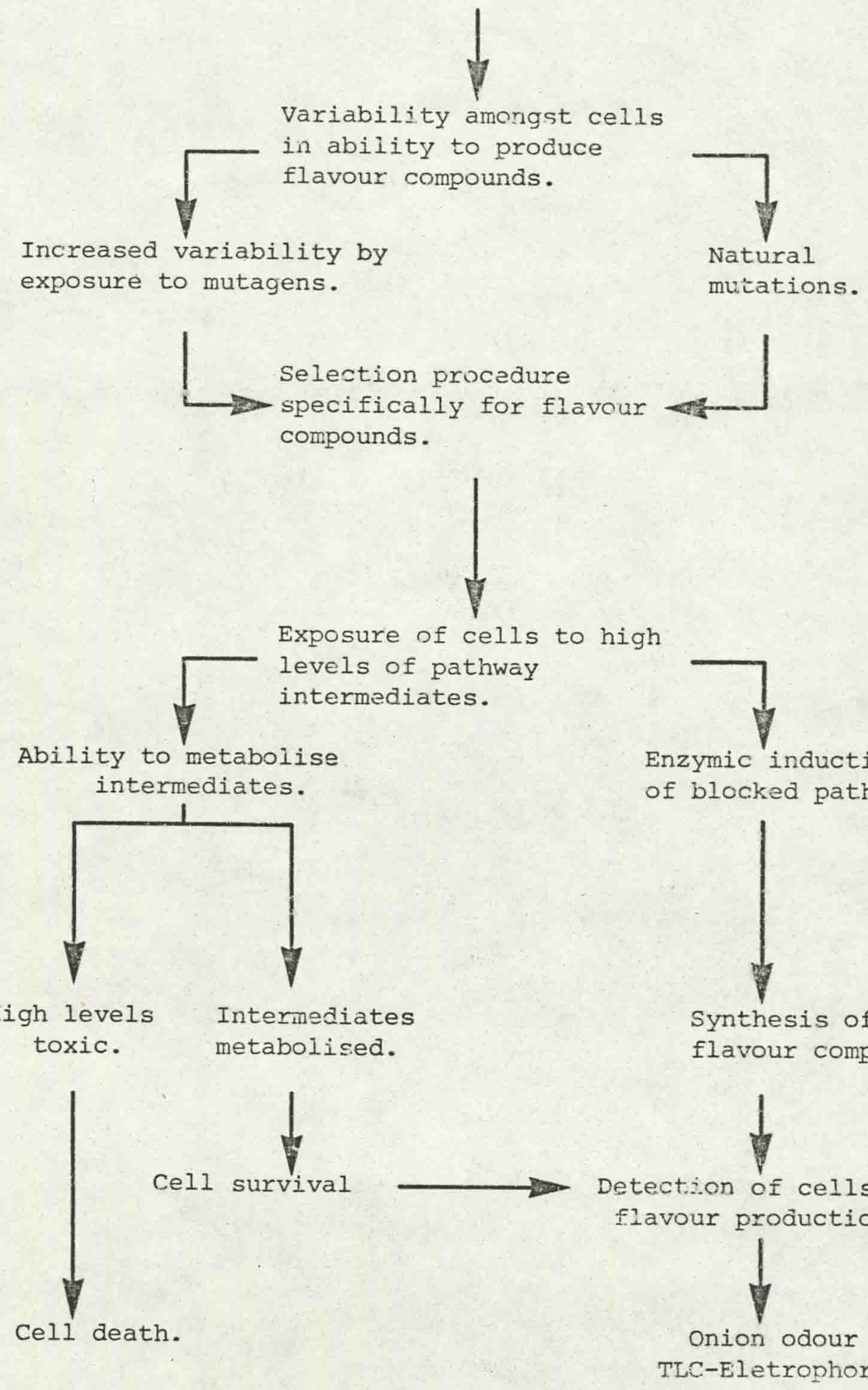
since they are similar enough in structure to be incorporated into a newly synthesised strand of DNA, whilst others, the frame-shift mutagens, cause the loss or addition of one or two bases in the DNA during replication or repair so that the resulting base code is nonsense (Drake 1970).

The third group of mutagenic agents consists of those compounds which cause a chemical change in one or more of the nucleic acid bases while they remain in situ in the molecule. In other words, these compounds act on resting DNA (Freese 1971) unlike the other mutagens mentioned above. For routine isolation of mutants, this group of agents which includes nitrous acid, NTG and ethyl methane sulphonate (EMS) is the most widely used and convenient. Nitrous acid was chosen for use in this investigation because it is a relatively harmless substance and the amount of mutagenesis can be controlled easily by varying the exposure times (Hopwood 1970). NTG was used because it is one of the most potent mutagens known and it produces a high proportion of mutants amongst the surviving cells with relatively little cell death. However, the main disadvantage of NTG was the extreme care required in handling the solution. Nitrous acid had previously been used for the production of bacterial mutants (Kaudewitz 1959) and also in the study of DNA polymerase as an agent for the deamination of the DNA bases, cytosine, adenine and guanine (Hoepfinger 1973). NTG was recently used for the production of streptomycin-resistant mutants of Euglena (Diamond and Schiff 1974) and mutant cells of Nicotiana tabacum which were resistant to base analogues (Lescure 1973).

Most studies involving the use of chemical mutagens and the selection of the resulting mutants were originally carried out with micro-organisms but in recent years similar techniques have been applied to cultured cells and tissues of higher plants (Street 1976). It is obvious that the methods involved are most successful if suspensions of single cells or small cell aggregates are used, since it is fairly certain that the cells of a small group have been produced by the division of a single cell and therefore they are likely to be genetically identical (Street 1976). Larger cell aggregates probably originate from more than one cell and so the cells of each group will not be genetically uniform. For this reason, it was necessary to develop a suspension culture from the onion callus material. Some of the mutagen-treated and control tissues were placed in liquid medium to select for clones with the ability to form a suspension culture. Suspension cultures are invaluable in this type of work since the cells can be treated with chemical mutagens and plated out onto agar for selection in the same way as micro-organisms are routinely handled. Similarly, the use of cell suspensions is necessary for the large scale culture techniques which are currently in use for the production of medicinally important compounds from cultures of micro-organisms.

The report which follows describes the preliminary experiments which were carried out in order to select a suitable experimental procedure for the routine production of mutated cells and the development of a rapid screening method for the selection of high yielding clones. The selection procedure used is presented in Fig. 7.1.

Fig.7.1 Selection procedure for high yielding clones.



7.2 Materials and Methods

7.2i Treatment of callus tissue with nitrous acid

Nitrous acid was generated by the addition of an appropriate amount of solid sodium nitrite to 0.3M sodium acetate buffer, pH 4.2, to produce a 1M solution. The nitrous acid was filter-sterilised using a Millipore filter and placed in sterile Erlenmeyer flasks. Five pieces of callus, each approximately 0.4g fresh weight were added to each flask. After exposure times of 0.25, 0.5, 1, 2, 3 and 4 hours, the acid was neutralised with an appropriate volume of filter-sterilised 1M NaOH solution which was accurately determined by titration. The callus pieces were washed several times in sterile distilled water and each piece placed in a McCartney vial containing 10ml standard nutrient medium. The material was incubated in darkness at 26°C and, at the end of each six week passage, growth was assessed by visual inspection and fresh weight measurements. Ten replicates were used at each sample time.

Callus tissue was exposed to 0.3M sodium acetate buffer, pH4.2, as a control. A further control was used in which material was transferred directly to fresh nutrient medium. Some of the treated material was transferred to 100ml Erlenmeyer flasks each containing 25ml liquid medium and the cultures grown in a 16 hour photoperiod at 26°C on a horizontal shaker at 100 rev. $min.^{-1}$

7.2ii Treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

Treatment with NTG solution (100 mg l^{-1}) was carried out as described for the nitrous acid treatment and the reaction terminated by washing the tissue several times in sterile distilled water to remove all traces of mutagen. However, in this case the solid mutagen was dissolved in liquid medium instead of distilled water and control tissue was incubated in liquid nutrient medium. Callus tissue three, six and nine weeks after subculture was used in this experiment.

7.2iii Development of a Selection Procedure.

Callus tissue was placed onto agar medium containing high levels (up to 100mM) of sulphate, valine, methacrylic acid and CPC. Details of this experiment were reported in Section 3.2i. Material was sampled at the end of each passage and growth assessed by measurement of fresh weight. Visual inspection of the callus determined the extent of tissue browning which occurred on transfer of material to physiologically high levels of intermediates. This was used as an estimate of cell death.

7.3 Results

Table 7.1 shows that treatment with nitrous acid or sodium acetate buffer prevented callus growth. The rapid decline in growth observed after the first passage was probably due to excessive water loss at the onset of senescence. Growth measurements after two and three passages showed a steady but slower decline indicating that the material had been killed by the treatments and was slowly drying out. There were no obvious differences amongst the treatments or between the control and mutagen-treated material. All callus samples were brown in colour with the intensity and extent of browning increasing with the length of exposure to either nitrous acid or the sodium acetate buffer. It is believed that tissue death occurred fairly rapidly after exposure to the treatment, probably due to the acidic nature of the medium. No healthy growing tissue was observed in any of the flasks after incubation of the callus on standard medium for up to eighteen weeks.

Table 7.2 shows the results of treatment with NTG. Nine week old callus tissue was discarded since cell death occurred after all treatments including the controls. Three week old tissue exposed to the mutagen for fifteen and thirty minutes showed some growth after one passage on standard medium but growth declined after two passages. Thus, treatment of six week old callus with NTG solution at a concentration of 100mg l^{-1} for up to thirty minutes were the conditions selected for further experiments. Material under these conditions showed a small increase in fresh weight after one hour's exposure to NTG but by the end of the second passage, growth had declined whilst those tissues exposed to NTG for fifteen and thirty minutes were still growing.

Table 7.1 Effect on callus growth of treatment with sodium acetate and nitrous acid solutions after one passage.

Exposure time (hours)	% increase in fresh weight		
	1M $NaNO_2$ in 0.3M sodium acetate, pH 4.2	0.3M sodium acetate, pH 4.2	Control
0.25	-23.52	-28.00	321.76
0.50	-24.21	-24.13	
1.00	-18.65	-28.59	
2.00	-18.65	-29.92	
3.00	-21.73	-23.67	
4.00	-15.46	-25.71	

Table 7.2 Effect of treatment with NTG on callus tissue of various ages after one passage.

Exposure time (hours)	% increase in fresh weight					
	NTG treatment			Control		
	Age of culture (weeks)			Age of culture (weeks)		
	3	6	9	3	6	9
0.25	17.37	13.92	-18.31	30.31	192.93	-30.62
0.50	1.95	51.67	-21.10	93.45	166.46	-26.15
1.00	-10.58	13.44	-24.68	100.98	63.10	-23.10
2.00	-26.38	-40.55	-21.55	42.14	173.66	-22.92
3.00	-30.22	-47.51	-28.38	159.97	184.75	-16.07
4.00	-30.29	-51.24	-22.22	37.58	201.81	-8.14

True suspension cultures were not obtained from either the mutagen-treated callus or from the controls. Very thin suspensions formed in some of the flasks but examination of the medium showed most of the free cells to be dead. However, much of the callus material remained in small clumps and did not break up into smaller cell aggregates or release viable free cells. Callus pieces were removed to fresh medium after three weeks incubation and the remaining liquid was divided between two flasks of fresh medium, but no further suspensions resulted from these inocula. No accurate assessment of fresh weight could be made since it was difficult to account for the amount of liquid medium on the callus pieces, although visual assessment estimated that little tissue growth occurred. In a further experiment, two mutagen-treated callus clones were used, one of which was of a friable nature whilst the other consisted of larger, compact pieces of tissue, but no difference was observed in the ability of either clone to form a suspension.

The method employed for the development of a mutant selection system was not entirely successful, probably due to the use of callus pieces rather than a suspension of single cells or cell aggregates. It was intended to select two levels of a particular intermediate, one of which allowed slight growth and the other which prevented callus growth. This would then provide a threshold level of intermediate on which to place mutagen-treated tissue. Callus tissues able to survive and grow on this threshold level were to be cultured for the production of significant levels of onion flavour compounds.

The presence of methacrylic acid in the medium at all levels prevented growth (Table 7.3).

Table 7.3 Effect on callus growth of high levels of methacrylic acid, valine and sodium sulphate in the medium.

Concentration (mM)	% increase in fresh weight					
	Methacrylic acid		Sodium sulphate		Valine	
	Passage 1	Passage 2	Passage 1	Passage 2	Passage 1	Passage 2
0	275.70		275.70		275.70	
10	-15.24	-17.70	211.52	244.03	32.60	0.57
20	-16.01	-20.01	189.92	239.43	7.88	0.15
50	-20.54	-12.45	78.83	121.63	-9.85	-20.06
70	-18.56	-17.52	52.61	80.84	-10.47	-4.88
100	-15.75	-16.08	26.28	30.97	2.03	-17.25

Significant growth occurred on all levels of sulphate but growth declined as the sulphate level increased (Table 7.3). However, after two passages on sulphate-containing medium the growth at each sulphate level was significantly higher than that recorded after only one passage. In other words, the tissue was apparently becoming habituated to the presence of excess sulphate in the medium and at the lower levels of 10mM and 20mM, growth approached that of the control.

Growth measurements for valine-fed callus (Table 7.3) indicated that there was a threshold level of valine between 20mM and 50mM which allowed callus survival but did not support growth. This was supported by observations that the callus on the lower levels of valine appeared to be healthy whilst the tissue on levels of 50mM valine and above showed increased amounts of brown, dry material.

The results of CPC incorporation into the medium are shown in Table 7.4. Significant growth, approaching that of the control, was obtained at all levels of CPC up to 20mM but this declined markedly at 30mM whilst 100mM CPC in the medium prevented growth. It was also demonstrated that growth declined as callus was transferred to a higher level of CPC but growth was maintained if the callus was transferred to fresh medium containing the same level of CPC. It is likely that the threshold level of CPC for callus survival is in the region of 60mM.

Table 7.4 Effect on callus growth of increasing levels of CPC in the medium.

Concentration (mM)	% increase in fresh weight			
	Passage 1	Passage 2	Passage 3	Passage 4
0	108.43			
4	92.27			
8	106.40			
12	88.96			
16	68.76	33.66(20mM)	-0.63(100mM)	0.03(100mM)
20	96.65	82.58(20mM)	3.62(100mM)	-5.43(100mM)
30	20.87	-12.12(100mM)	-20.54(100mM)	
100	-6.91	-7.96		

7.4 Discussion

The results of the experiments reported here showed that the treatment of callus tissue with nitrous acid was unsuccessful in that all the cells died. This was most likely to be due to the acidic nature of the medium since the control tissue was also killed. Badr et al. (1972) studied the effects of nitrous acid on the cell cycle and mitotic apparatus of *Vicia faba* cells and concluded that the mutagen had damaging effects on both of them. They classified the chemical as a spindle, chromosome and prometaphase poison, and yet the concentrations used were much lower than those often used in the routine generation of mutant cells. It was also reported by Tessman (1962) that nitrous acid not only produced point mutations due to the deamination of the DNA bases, but it also induced large deletions which were lethal.

Although suitable conditions for routine NTG treatment were selected from the preliminary experiments which were carried out, no mutant clones were isolated. The selection procedure was not available at the time of NTG treatment and so the callus was placed on standard medium to 'bulk up' the tissue before selection. However, although small patches of healthy, growing tissue were present amongst the brown, senescing material, this healthy callus soon died. This seemed to be because the cells were unable to receive nutrients from the medium except in those rare cases where the healthy cells were in contact with the nutrient agar. This was further complicated by the fact that healthy callus could not be separated from brown tissue and removed to fresh medium because

the callus pieces were too small to grow. It is well known (Caplin 1963, Yeoman and Macleod 1977) that there is a minimum size of callus inoculum below which the cultured tissue cannot survive.

Ideally, the NTG treated callus should be placed directly onto selecting media. It is suggested that for the selection of high yielding clones of onion tissue, the callus should be placed on medium containing valine. Although no onion odour was detected when valine-fed callus was crushed, the tissue was able to grow on the valine supplemented medium. It is believed that the effect of valine on the biosynthetic pathway may be slow since valine is metabolised slowly even in actively growing tissue such as onion shoot tips (Chapter 3). It is possible that valine will have the same effect on the synthesis of Pren Cy SO as exogenously supplied CPC, Pren Cys and Pren Cy SO but with a much longer lag phase before Pren Cy SO can be detected. If this is so, mutant or variant strains of callus tissue may be selected by estimation of their growth on media containing threshold levels of valine.

CPC at different concentrations was also shown to affect callus growth but since the synthesis of CPC from methacrylic acid and cysteine was demonstrated to be a 'weak link' in the biosynthetic chain (Chapter 3) it would be more suitable to supply a compound which is present in the pathway before this point. However, this selection procedure is not strictly specific for flavour precursor synthesis since the pathway intermediates may also take part in other metabolic pathways, although labelled

intermediates, when fed to onion tissue, were rapidly converted to Pren Cy SO. Also, the advantage of using cultured tissue as a source of variability was outweighed by the difficulties encountered in obtaining representative measurements. In many cases, much variation was recorded for control callus which then made assessment of the effects of supplemented media very difficult.

The use of callus material was not entirely satisfactory because any mutated cell could be masked by the majority of normal cells and so the growth of a mutant cell could not be detected readily by visual observation or measurement of fresh weight. Consequently, these systems would be much more successful if used with cell suspension cultures. Selby and Collin (Unpublished results) were unable to produce cell suspensions from onion callus using enzyme solutions, varying calcium levels in the medium, different agitation speeds of the liquid cultures and the presence of glass beads in the medium. Similarly a clone capable of producing a suspension was not formed after mutagen treatment of onion callus regardless of the nature and degree of friability of the initial material.

A possible alternative to cell suspensions lies in the use of cultured protoplasts. The techniques of protoplast isolation and culture are now being used for the study of cell wall regeneration (Pojnar et al. 1967, Willison 1976), fusion of isolated protoplasts for somatic hybridization of plants (Power et al. 1970, Giles 1974), and the uptake of macromolecules for

studies on virus infection (Takebe and Otsuki 1969, Zaitlin and Beachy 1974) and have been reviewed by Cocking (1972, 1975), Eriksson et al. (1974) and Evans and Cocking (1977). Vardi et al. (1975) reported the isolation of protoplasts from Citrus sinensis and the subsequent mutagenic treatment of the cultures with X-rays and EMS for the induction of embryoid regeneration. Thus, isolated protoplasts may be important in some situations where cell suspensions are difficult to induce and maintain.

As early as 1957, Vreugdenhil reported the effects of environmental factors on protoplasts of Allium cepa whilst later work by Bawa and Torrey (1971) described the isolation of protoplasts from onion root callus. They also reported the presence of nuclear division in the protoplasts although true mitosis and cell division was not observed. Such techniques require further investigation and modification before they can be used to produce high yields of isolated protoplasts but they may have an important role in situations, as found in the onion cultures, where cell suspensions are not formed readily.

One of the disadvantages of using diploid cells for mutagenesis work is the fact that a mutation would have to be dominant before it could be detected, since the effect of a recessive gene would be masked. For this reason, the use of pollen and anther cultures (Nitsch 1975) is becoming more important today for the induction and direct selection of mutant cell lines (Chaleff and Carlson 1974, 1975) since they are the source of large, homogeneous populations of haploid cells in which each cell contains a single

copy of the genetic information (Sunderland and Dunwell 1977). The use of haploid cell cultures which can be subjected readily to mutagenesis and selection should provide a useful tool for the genetic modification of higher plant cell cultures (Nelson and Burr 1973) and the isolation of high yielding cell lines. The production of haploid cells from pollen and anther cultures and the potential for their use in the study of higher plant genetics has been described by Devreux (1970) and Street (1976).

As far as is known, no anther or pollen cultures have as yet been produced from the onion plant but the culture of haploid onion cells would facilitate induction and selection of high yielding strains. Thus, there are possible alternatives to the use of callus material in the isolation of cells able to synthesise large amounts of onion flavour compounds, and in the light of similar work on other industrially important species (Misawa 1977), selection of high yielding variant strains is likely.

CHAPTER 8

GENERAL DISCUSSION

Since details of the first successful continuous culture of higher plant cells were reported independently but simultaneously by Nobecourt, White and Gautheret in 1939, the techniques of plant tissue culture have been used for a wide range of experimental applications (Murashige 1977). These include cellular metabolism and differentiation studies, plant breeding and evaluation of herbicides and plant growth regulators. However, the important application for the purpose of this investigation is the use of tissue cultures for the production of economically important compounds (Staba 1963, 1977, Turner 1971, Constabel et al. 1974, Butcher 1977, Tabata 1977). Examples of these compounds include pharmaceuticals such as alkaloids (Chan and Staba 1965, Neumann and Muller 1971, Veliky 1972), antibiotics (Mathes 1963, Campbell et al. 1965, Khanna and Staba 1968) and steroids (Heble et al. 1971, Kaul et al. 1969), fragrant oils of interest to the perfume and cosmetic industries and flavourings such as mint oil (Wang and Staba 1963), mustard oil and onion oil (Selby and Collin 1976). However, as mentioned earlier, the majority of cultured tissues produce the same secondary products as those found in the intact plant but at a much lower level. Thus, cultured material cannot be used as a source of industrially important compounds unless the tissues can be induced to synthesise these products in significant amounts.

The present report describes an investigation which was carried out using onion callus cultures to determine the reasons for reduced secondary compound synthesis in cultured tissues and the approaches

used to increase synthesis of these products.

The experiments reported in Chapter 3 showed clearly that onion callus was able to incorporate radioactive intermediates into Pren Cy SO, the main flavour precursor of onion tissues. In other words, cultured tissue was able to synthesise the desired secondary product although only at very low levels. However, later experiments indicated that the amount of secondary product synthesis could be significantly increased by supplying a low concentration of the required compound. Thus, the callus can be induced to synthesise large amounts of Pren Cy SO by feeding much smaller amounts of the same compound or one of its immediate precursors such as CPC or Pren Cys. Pren Cy SO can be readily obtained from fresh onion material (Granroth 1970) and so for industrial application of this work, low levels of this compound could be fed to callus to induce synthesis of economically useful amounts of this flavour precursor. The use of naturally-occurring compounds extracted from onion would be preferable to synthetic precursors because the latter usually consist of a mixture of cis and trans isomers. Naturally-occurring compounds have the trans configuration, although the metabolism of the cis isomer of CPC has been demonstrated for onion tissues (Granroth 1970).

Although callus growing on valine-supplemented media did not produce the characteristic onion odour when the tissue was crushed, it is possible that the callus would be able to metabolise valine to Pren Cy SO. The lag period which occurred between the supply of intermediates and the appearance of Pren Cy SO was found to

increase according to the number of biosynthetic steps required to convert the intermediate to the end product. Thus, it is believed that valine would operate in the same way as exogenously supplied CPC or Pren Cy SO and induce Pren Cy SO synthesis, although in this case a much longer lag phase would be expected. Direct feeding of valine solution to the callus instead of its incorporation into the medium would be preferable and may reduce the lag period. Further investigation of this hypothesis is required, but if the system can be shown to operate in callus, whereby the supply of a low concentration of valine solution to the tissue induces Pren Cy SO synthesis, then it may be of use to the flavour industry. Valine is a relatively inexpensive chemical to supply and the Pren Cy SO so produced could be substituted in situations where onion powder is used at the present time. Similarly, the use of small amounts of Pren Cy SO to induce increased synthesis of this compound should be less costly than the extraction of flavourings from onion bulbs.

The regulation of secondary product formation in cultured cells has been controlled in many cases by the supply of precursors as described for onion cultures in this investigation. Occasionally it was found that whilst indirect precursors did not increase the yield of the required product, more direct precursors were able to do so (Zenk et al. 1975). Examples of successful secondary product synthesis as a result of precursor feeding include the stimulation of alkaloid production in Datura stramonium cultures

by exogenous ornithine and phenylalanine (Chan and Staba 1965) and in Ruta graveolens suspension cultures by quinolone (Steck et al. 1973). One important application of precursor feeding is the biotransformation of key intermediates to medicinally important compounds such as the alkaloids harman and norharman (Veliky and Barber 1975) and the conversion of progesterone to pregnanolone by cultured cells of Digitalis purpurea (Furuya et al. 1971a, 1973). However, the supply of precursors to cultured cells does not always result in an increase in the synthesis of secondary compounds. Several experiments have shown that the amount of precursor supplied to the culture may be critical (Tabata 1977). For example, a single large dose of hydroquinone fed to Datura innoxia cells resulted in cell death whilst a small amount fed daily increased the yield of the required compound, arbutin (Tabata et al. 1976). In the onion callus cultures used in this investigation, a high level of CPC caused some cell death whilst a much lower level applied directly to the tissue stimulated flavour precursor synthesis. Similar results are expected if small amounts of valine are fed directly to the callus.

The second important factor which can be used to manipulate secondary product synthesis in cultured cells is the initiation of morphological differentiation. It has long been known that organisation of the callus tissue of many species, into roots, shoots and plantlets, is paralleled by the synthesis of significant amounts of those secondary compounds characteristic of the intact

plant. Examples include the restoration of cardenolide synthesis in redifferentiated shoots of Digitalis purpurea callus (Hirotani and Furuya 1977), alkaloid production in regenerated plantlets of Datura innoxia callus (Hiraoka and Tabata 1974) and Coptis japonica cultures (Ikuta et al. 1974). Similarly, in the investigation reported here (Chapter 4) evidence was presented to show that regenerated roots and shoots of onion callus contained the same pattern of flavour precursors as the corresponding organs of the whole plant, although the relative amounts of each compound may have varied. Further quantitative analyses are required to clarify the situation. However, the differences in lachrymatory potency between callus-produced organs and those of the bulb or seedling would suggest important differences in flavour precursor levels. The increased water content of cultured tissues may have had an effect in diluting the flavour precursors but this would be insufficient to account for the very weak odour which was produced when regenerated shoot material was crushed.

The phenomenon of secondary product synthesis in organized callus tissues is of potential use for the production of such compounds in economically useful quantities. The ultimate aim in industrially orientated studies is to isolate the conditions under which morphogenesis and development occur so that chemical differentiation may be achieved in the absence of morphogenesis

for the commercial production of secondary plant products. In the absence of such a system, it is possible that a two stage process may be employed in which cells are first allowed to grow and divide rapidly before induction of partial differentiation, so that secondary products can be synthesised and subsequently harvested (Mandels 1972).

Several factors which influence or control morphogenesis have also been found to exert effects on the biochemical expression of the cell, eg. hormones (Skoog and Montaldi 1961, Furuya et al. 1971b, Gamborg et al. 1970), medium composition (Amorim et al. 1977, Dougall 1977) and light (Corduan and Reinhard 1972, Brunet and Ibrahim 1973). However, it is still uncertain if these factors exert a direct effect on secondary product synthesis or whether the effect is mediated by the morphogenic response of the cells which subsequently affects secondary metabolism. Evidence is available which showed that starch accumulation was detected in cells of tobacco callus before any observable organized development, with accumulation occurring to a greater extent in those cells which ultimately gave rise to organ primordia (Thorpe and Murashige 1968). Gibberellic acid treatment was found to prevent starch accumulation and organization suggesting that the two phenomena were associated. Similar associations may exist between organized tissues and secondary product synthesis but as yet, no evidence has been presented to support this hypothesis.

The volume of evidence in the literature supporting the synthesis of secondary products on initiation of morphogenesis in cultured tissues accentuates the importance of tissue organization. Murashige (1977) drew attention to the fact that cells in culture are not undifferentiated as commonly believed. They are in fact, partially differentiated or at least structurally modified (Constabel et al. 1974) but usually unorganized and it is this absence of organization which is thought to result in failure of the cultured cells to produce secondary metabolites.

In fact, in the intact plant, active meristem areas do not normally show deposits of secondary metabolites such as alkaloids, flavonoids and terpenoids (Constabel et al. 1974). However, as soon as the meristematiccells become differentiated and organized into cell types, secondary products often appear.

Cultures may become organized by production of roots, leaves and buds, but also by the formation of meristematic centres and vascular and lacticifer elements which are much more difficult to detect. Thus, it is possible that some differentiation was present in those cultures reported to produce high yields of secondary metabolites. This is clearly illustrated by the only known case of volatile oil production by cultured tissue (Corduan and Reinhard 1972). Callus cultures of Ruta graveolens were reported to produce the characteristic oil of the whole plant. However, callus tissue of this plant is a highly organized tissue and possesses schizogenous canals below the callus

surface in which the oil accumulates.

Investigation at the cellular level suggested that the onion volatiles are concentrated in special oil cells which are associated exclusively with the vascular bundle sheaths (Becker and Schuphan 1975). However, the staining techniques used in these experiments were not specific for the onion flavour components. Radioactively-labelled sulphate was also shown to be localized in individual cells around the vascular bundle but this could not be used as a direct measure of flavour compound accumulation. They concluded that the volatile oils were associated with specialised cells which were produced very early in ontogenesis, although no free flavour compounds could be detected in the dormant seed.

In some cases, tissue organization may be important if the precursors and the secondary product are synthesised in different tissues or organs of the plant. Thus, the precursors would require transportation to the site of synthesis of the final product (Butcher 1977). This could explain the absence of secondary product synthesis in the callus cultures of those species which do not require special structures such as oil glands in the intact plant. The suggested site of synthesis for the onion flavour precursors was the stem (Freeman 1975) since it forms a junction between the two pathways of photosynthate from the leaves and inorganic ions from the roots. However, the flavour compounds are apparently transported from the stem to

other parts of the plant for storage since the work of Saghir et al. (1965) and Freeman (1975) showed that the flavour compounds were present in all parts of the onion plant. Transport of the secondary product away from the site of synthesis in the intact plant may prevent feedback inhibition of the biochemical pathways involved. Thus, such inhibition may occur in callus tissues if the product remains at the site of synthesis, thereby repressing further synthesis of the secondary product.

Secondary metabolite synthesis or accumulation in the plant is often associated with specialised cells or organs such as oil glands (Carew and Staba 1965, Constabel et al. 1974, Tabata 1977). One of the best examples of this is the mint plant where mint oil is excreted and accumulated by glandular hairs and scales in the epidermis. Mint oil components were not detected in cell cultures unless the structural features of the gland cells were present (Wang and Staba 1963). Similarly, Hart et al. (1970) found that regenerated shoots of Pogostemon cablin cultures did not produce the sesquiterpene components of Patchouli oil present in the whole plant. They attributed the lack of volatile oil production to the absence of glandular trichomes in which the oil usually accumulates. It was suggested that end product inhibition may occur if trichomes were not present in cultured tissue to act as a sink to remove the newly synthesised sesquiterpenes, thus resulting in undetectable levels of product.

Consequently, compartmentation either at the level of different cell types or at the subcellular level, may be an important mode of

regulation for various biosynthetic pathways (Hahlbrook 1977). It is likely, therefore, that features such as spatial arrangement of enzymes, the presence of specific organelles, compartmentation of enzyme and substrate and locations available for deposition may all determine whether a secondary compound is produced (Butcher 1977). For example, in the absence of compartmentation in cultured cells it is possible that any secondary product formed might be immediately degraded by specific enzymes. Similarly, the absence of a particular organelle may result in reduced secondary product synthesis because no site of deposition or accumulation is available.

The specialised vesicles found in the onion bulb cells (Chapter 5) are believed to be the sites of accumulation of the onion flavour compounds. It is possible that the absence of such vesicles in callus cells results in reduced flavour compound synthesis due to the lack of a suitable accumulation site for these compounds. For this reason, it would be of interest to examine the ultrastructure of callus cells as they become differentiated and organized to form functional organs, to determine whether the development of secondary product synthesis is associated with vesicle formation or other structural modification. A similar investigation was carried out on cells of Atropa belladonna callus (Simola 1972) where it was shown that accumulation of alkaloids in small vacuoles was the first sign of differentiation in the cells. However, many fully differentiated

cells did not contain significant amounts of these products.

Stored Pren Cy SO was not detected in any fraction of the onion homogenate (Chapter 6), presumably because it was degraded by alliinase as soon as the cells were ruptured. However, most of the enzyme activity should have been repressed by storage of the tissue at $4^{o}C$ overnight before the material was homogenised.

It is very difficult to identify the material which was stored in the special vesicles reported to be present in onion cells because as far as is known, there is no specific histological stain for the flavour compounds. An alternative method for the identification of these compounds would be to isolate specific organelles from tissue homogenates under conditions designed for each organelle. This could be followed by assays specifically for detection of the alkyl and alkenyl cysteine sulphoxides. However, membrane bound structures such as lysosomes, vesicles or central vacuoles are particularly susceptible to damage during the homogenisation and centrifugation procedures. This would make a pure fraction of undamaged organelles very difficult to achieve. Therefore, most of the evidence for the association of the flavour compounds with specialised vesicles in the onion cells remains circumstantial. However, it may be possible to locate Pren Cy SO synthesis and accumulation in onion cells by examination of the bulb tissue by micro-autoradiography, after the intact plant had been supplied with radioactive intermediates such as ^{14}C-cysteine.

Much evidence has been presented to show that cultured cells of the higher plant are morphologically totipotent and that

given suitable conditions of growth, such cells are capable of differentiating and forming organized tissue. This organized tissue is then able to regenerate into roots, shoots and plantlets. Thus, it is reasonable to suppose that each cell is also biochemically totipotent so that in the presence of the correct stimuli, it should be possible to reproduce the biochemistry of the intact plant (Krikorian and Steward 1969). However, it seems that the morphology and biochemistry of the cells differ by virtue of the position of each cell in the plant body and the restraints imposed on the cell by its immediate environment and the extrinsic forces which act on that cell (Steward et al. 1964, 1967). Thus, the process of differentiation of any particular cell in the whole plant has been described as a process whereby the genetic information present in the zygote is gradually repressed but structurally unchanged (Vasil and Hildebrandt 1966). Consequently the process of de-differentiation which occurs during callus initiation from higher plant tissue explants involves the removal of gene repression, sometimes referred to as deprogramming (Yeoman and Aitchison 1973). This is such that mature cells lose the inhibition of gene action which operates in differentiated cells. In other words, some of the physiological control mechanisms for organized development are eliminated by the isolation associated with 'in vitro' culture techniques.

By application of suitable 'in vitro' physiological controls which either resemble or duplicate the controls operating 'in vivo', it should be possible to re-direct the undifferentiated cells to

begin the sequence of developmental steps leading to differentiation and organization. The direction of development of a particular cell is therefore determined by the interaction of chemical and physical influences from its environment on the intrinsic genetic control mechanisms of that cell (Steward et al. 1967). For the purpose of secondary product synthesis in cultured tissue systems, the ultimate aim is to achieve biochemical differentiation without the necessity of reproducing the entire morphogenesis (Krikorian and Steward 1969, Street 1973).

The ability of the plant to synthesise and accumulate amino acids in significant amounts is a genetically controlled characteristic (Bell 1976). It appears that the different patterns of amino acid accumulation in different species reflects differences in the degree to which particular genes are 'switched on' (Fowden 1974). Consequently, it seems that in the onion plant the necessary genes are 'switched on', thus allowing the operation of the biosynthetic pathway and resulting in the accumulation of large amounts of secondary product. However, in the onion callus tissue, the enzymes necessary for the biosynthesis of Pren Cy SO are present but they are apparently operating in the fully repressed state. Similar situations have been described for other tissue culture systems where it was suggested that the rapidly growing cultured cells have sufficient enzymic activity for their biosynthetic needs and that such a level of activity is at, or closely approaching, the fully repressed level of enzyme activity (Dougall 1977). In other words, it was assumed that the end products of the metabolic pathways in question are maintained at a

level sufficient to achieve almost complete enzymic repression.

This hypothesis could be significant in the case of the onion callus tissue. For example, callus cells lacked the specialised vesicles of onion cells in which it is believed the secondary products of the onion were accumulated. Thus, when the callus cell synthesised the secondary product, it was not removed from the site of synthesis, and so the small amount of this compound that was produced caused feedback or end product inhibition of the biosynthetic pathway enzymes.

However, when the callus cells differentiated, the mechanism for flavour precursor synthesis was 'switched on', perhaps by the de-repression of the genes controlling the biosynthetic pathway. This may also be associated with the appearance of special vesicles in differentiated callus cells, but this has not yet been demonstrated.

It has been suggested that once a particular secondary biosynthetic pathway has been opened up by inactivation of repressor molecules or inactivation of the regulator genes responsible for synthesis of the repressors, then the pathway can be intensified by modification of the cultural conditions (Street 1973). Such a system may be of use for the onion cultures. For example, if the pathway for Pren Cy SO synthesis can be opened up by supplying very small amounts of the compound itself or one of its precursors, then the onion cultures could be used for the

production of high yields of secondary products. However, to prevent feedback inhibition of the enzymes by the final product, the product must be secreted into the surrounding medium from which it could be extracted. It seems that this may have occurred in the onion callus because the level of Pren Cy SO which was produced, increased over a two week period. However, no analysis was carried out on the agar medium and so this requires investigation. The presence of white deposits on the surface of the onion callus which was growing on CPC-supplemented media may indicate secretion or excretion of some product but this also requires further investigation.

One of the main disadvantages of using cultured material for industrial purposes is the fact that most cultured tissues are unstable if continuously subcultured for long periods. This instability is reflected in the changes which occur in the morphological and physiological expressions of the cells (Torrey 1970). For example, cultured cells tend to become progressively more friable, lose their capacity to initiate organized development (Smith and Street 1974) and show altered growth rates. Continuous subculture may also affect the level of secondary compounds produced. It was shown for cultures of Nicotiana rustica that the level of nicotine removed from the callus tissue soon after callus initiation rapidly declined to trace amounts in parallel with the decline in root regenerating ability (Tabata and Hiraoka 1976). Such changes have been attributed to a predominance of

polyploid cells in the culture (Demoise and Partanen 1969, Kao et al. 1970), particularly aneuploids, which tend to increase with the length of the culture period (Torrey 1977). This may occur very rapidly as observed in callus of Citrus limon where the percentage of diploid cells in the culture declined from 100% to 33% by the end of three, four week passages (Murashige et al. 1968). Many cultured cells also show chromosomal aberrations such as chromosome bridges or fragments (Novak 1974).

Thus, cell cultures are not homogeneous populations as was once believed. In fact, they consist of a mixture of cell types. In many cases, the basic genome remains unchanged whilst the expression of the available genetic information is modified or reduced in response to a particular set of culture conditions. This can be illustrated by examples of cultured cells in which particular strains can be selected and grown in isolation. These include the selection of maize callus cultures resistant to the toxin produced by the plant pathogen Helminthosporium maydis (Gengenback and Green 1975) and cultured cells of Acer pseudoplatanus resistant to p-fluorophenylalanine (Gathercole and Street 1976).

However, the instability which occurs in cultured cells (Sheridan 1975) also provides an opportunity for genetic manipulation of higher plant cells and the subsequent selection and characterization of mutants (Street 1975, 1977d). This is of

particular importance for the isolation of biochemical mutants for the synthesis of secondary products in culture (Widholm 1977).

Although the experiments reported here (Chapter 7) for the selection and growth of naturally-occurring and mutagen-induced onion cell lines with the capacity to produce high yields of flavour compounds were unsuccessful, a selection procedure was described which may be of use in future investigations. The main problem encountered in this work was in the use of callus material. It is essential that a suspension of single cells, isolated protoplasts or small cell aggregates is developed from onion callus cultures if the selection of high yielding clones is to be successful. A cell suspension would also be of value in an industrial situation because it is not economically feasible in terms of time, space and expense, to culture callus material on agar medium. Suspensions of onion cells could then be cultured for the production of flavour compounds using large scale fermentation and batch culture techniques. Several workers have suggested the possibility of controlled production of important compounds on a commercial scale, but this has not yet been achieved (Mandels 1972, Constabel et al. 1974, Street 1977a, 1977c).

Several possible functions have been suggested for the occurrence of secondary products in higher plant tissues, one of the most widely accepted being the role of plant protection (Hegnauer 1975, Mitschner 1975, Swain 1977). Several reports in the literature describe the antimicrobial effects of extracts from

various members of the Allium family (Abdou et al. 1972). For example, aqueous extracts of Allium sativum were found to inhibit the growth of Candida albicans (Barone and Tansey 1977) and many other fungal species (Appleton and Tansey 1975, Tansey and Appleton 1975) whilst aqueous extracts of fresh garlic or garlic powder have been used to control foot rot of Phaseolus vulgaris caused by the fungus Fusarium solani f. sp. phaseoli (Russell and Mussa 1977). It has also been shown that a much lower fungal colonisation occurred on the rhizoplane of Allium cepa and Allium sativum than in other crop plants (Parkinson and Clark 1964).

The role of flavour compounds in the onion as protective agents could explain the repressed synthesis of these compounds in callus. The cultures would not require protective compounds because the tissues are maintained in sterile conditions. Further experiments could be carried out in which the onion callus was grown under normal, sterile culture conditions with specific fungal species which are normally found associated with the onion plant, for example, the vesicular-arbuscular mycorrhizal fungi Glomus fasciculatus and Glomus mosseae (Schoknecht and Hattingh 1976). Thus, it would be expected that callus growing under such conditions might begin to synthesise increased levels of flavour compounds. The main drawback to this type of experiment would be in obtaining fungal species capable of growth in the culture conditions.

It is interesting to note that an alliinase enzyme has been isolated from the fungus Penicillium corymbiferum (Durbin and Uchytil 1971). The function of this enzyme may be the degradation of the alkyl cysteine sulphoxides found in members of the Allium family, the Cruciferae and other related species, to render the compounds less harmful to the growth of this fungus.

Secondary compounds have also been ascribed the function of waste products, particularly since they are often separated from the rest of the cell or tissue in particular organelles or specialised cells respectively. However, this seems highly unlikely in view of the diversity of structure of these compounds and the range of concentrations and plant species in which they are found (Fowden 1972).

Conversely, plant cells have been shown to conjugate foreign compounds with glutathione (Barz 1977). Similarly, it could be argued that the secondary products of the onion are bound with glutamine to form γ-glutamyl peptides which either renders them harmless or effectively removes them from the primary metabolic processes of the cells. This could explain the occurrence of large amounts of γ-glutamyl peptides which are formed from the flavour compounds in the onion plant (Virtanen 1965, Whitaker 1976).

It is possible that the flavour compounds of the onion originated as waste products which, by virtue of their anti-microbial properties, conferred a selective advantage upon the plant. Such compounds could be stored in the bound form as peptides which do not have antimicrobial activity, until such times

when fungal or bacterial attack was most likely, such as at the onset of germination. This could explain why the transpeptidase enzymes are only active in the germinating seed and sprouting bulb, but are absent from the dormant organs.

The γ-glutamyl peptides of onion tissues have been described as storage compounds for nitrogen (Whitaker 1976) because they are rapidly metabolised at the onset of bulb sprouting and also because they are derivations of glutamine which represents an active substance in nitrogen metabolism (Virtanen 1965).

Experiments on the sulphate nutrition of onion (Freeman and Mossadeghi 1970) and garlic (Freeman and Mossadeghi 1971) have shown that the flavour strength and lachrymatory potency of the vegetables increased in proportion to the amount of sulphate supplied. Thus, it was concluded that the flavour precursor compounds were not indispensable for plant growth (Freeman and Whenham 1976) and that in conditions where a low level of sulphate was available, this would be used primarily for the synthesis of essential amino acids and proteins (Thompson et al. 1970).

8.1 Future Prospects.

1. Several experiments could be carried out to continue the work described in Chapter 3. For example, trans-Pren Cy SO could be extracted from mature onion bulbs and used for the feeding experiments using callus material. This would determine any differences between the naturally-occurring and the synthetic compounds in the effects they exert on the synthesis of Pren Cy SO in callus. Similarly, valine could be supplied directly to the callus as a weak solution to determine if this will stimulate Pren Cy SO synthesis. It would also be necessary to analyse the agar medium for the presence of Pren Cy SO to ensure that the product does not accumulate in the cells and cause enzyme repression.

Supply of ^{14}C - Pren Cy SO to the callus followed by analysis by TLC - electrophoresis and autoradiography would show whether the Pren Cy SO was degraded or bound with glutamine to form γ-glutamyl peptides.

2. Further investigation of differentiating callus cells by electron microscopy would clarify the situation regarding the specialised vesicles found in onion cells.

Location of the biosynthetic pathway and the sites of accumulation of Pren Cy SO, in isolated, subcellular fractions would help to locate the intracellular sites of synthesis and accumulation of Pren Cy SO in the intact plant. The procedure could then be applied to callus homogenates to discover if there

is any fundamental difference between the two tissues. However, the biosynthetic pathway must first be shown to operate in crude homogenates of onion tissues.

It would also be of use if a system could be developed for the isolation of intact lysosomes and vacuoles from onion tissues. This would enable identification of the contents of these organelles in the onion cell.

3. Development of a suspension culture from onion callus cells is vital for the selection of naturally-occurring and mutagen-induced variants or mutants. It would also be an advantage if the culture conditions could be modified so as to stimulate a faster growth rate in the onion callus cultures.

4. Finally, growth of individual fungal species in culture with the onion callus may suggest a possible role in plant protection for the flavour compounds of the onion plant.

APPENDIX 1

Synthesis of Cysteine Derivatives

i S-Propyl-L-Cysteine

7.85g cysteine hydrochloride were dissolved in 100ml of 2M NaOH and 130ml ethanol were added. 10ml 1-bromo-propane (propyl bromide) were added and the mixture left for 24 hours at room temperature with stirring.

After acidification to pH2.0 with concentrated HCl, the mixture was evaporated to dryness under vacuum. The resulting solid was washed with 100ml then 50ml of ethanol and the combined ethanolic extracts evaporated in vacuo.

The precipitate was dissolved in a minimum volume of distilled water. On addition of concentrated ammonia solution to pH4-5, the product was precipitated. After washing with water, the yield was 3.7g solid with a melting point 230-233°C.

ii Other S-Alkyl-L-Cysteine compounds

S-allyl-L-cysteine was synthesised as above from cysteine hydrochloride and allyl bromide, and S-ethyl-L-cysteine was synthesised from ethyl bromide and cysteine hydrochloride. S-methyl-L-cysteine was purchased from Sigma Chemical Company.

iii S-Propyl-L-Cysteine Sulphoxide

500mg S-propyl-L-cysteine were dissolved in 10ml glacial acetic acid with slight warming. The mixture was cooled to

12°C and 0.43ml 30% hydrogen peroxide was added with stirring. After 4 hours at room temperature, the mixture was reduced to dryness under vacuum.

The crystalline residue was dissolved in 5ml distilled water and the solution mixed with hot acetone till turbid. After cooling, 310mg fine needles precipitated which, after recrystallization from acetone, melted between 161 and 163°C, decomposing and foaming.

iv Other S-Alkyl-L-Cysteine Sulphoxides

S-allyl-L-cysteine sulphoxide, S-methyl-L-cysteine sulphoxide and S-ethyl-L-cysteine sulphoxide were synthesised by the method above, using the appropriate S-alkyl-L-cysteine.

Both the methods outlined above are modifications of those described by Stoll and Seebeck (1949).

v S-Propenyl-L-Cysteine

3g di-propenyl-L-cysteine (S-allyl-L-cysteine) were dissolved in 200ml dimethyl sulphoxide which had been dried by distillation. 3g potassium-t-butoxide were added and the mixture left in a stoppered flask for 18 hours at room temperature with stirring.

The cooled solution was treated with 400ml iced water and 10ml glacial acetic acid, then the resulting mixture passed through a column of 65g Amberlite IR 120 (H^+). The product was eluted with 400ml 2M NH_4OH. On concentration

of the eluate to 25ml, 1.24g crystalline product was recovered. Further concentration of the mother liquor to 10ml, with the addition of 3ml ethanol, produced 0.94g product.

Recrystallization of the combined products from aqueous ethanol yielded 1.8g of the pure compound which had a melting point of 179-180°C.

This method is a modification of that described by Carson and Wong (1963).

vi S-Propenyl-L-Cysteine Sulphoxide

3.5g S-propenyl-L-Cysteine were dissolved in 250ml water then cooled to 5°C. 2.65ml 33% hydrogen peroxide were added over 8 hours at a rate of 0.4ml $hour^{-1}$. The mixture was allowed to warm up to room temperature then left for 18 hours at room temperature with stirring.

The mixture was evaporated in vacuo to yield an oily solid which was then dissolved in 7ml distilled water. On the addition of 110ml ethanol, a white solid was precipitated. After 2 days at 4°C, centrifugation of the mixture yielded 2.7g solid.

This method is a modification of that described by Price and Snyder (1962) and Carson and Boggs (1966).

vii S-(β-Carboxy) Propyl-L-Cysteine (CPC)

3.5g cysteine hydrochloride were dissolved in 4.95ml methacrylic acid and 5ml water and the pH adjusted to

7.5 with 2M NaOH.

After 30 hours at room temperature under nitrogen, the reaction with sodium nitroprusside was positive. Heating for 2 hours in a water bath at 70°C resulted in the mixture being negative to the nitroprusside test.

Following acidification to pH2.0 with hydrochloric acid. the mixture was vacuum dried. The solid was dissolved in 12ml hot distilled water, then 5ml saturated sodium acetate were added. After several days in the cold, 1.83g white crystals were obtained (melting point 197°C). Two recrystallizations from small volumes of water resulted in a pure product with a melting point of 198°C. This method was a modification of those described by Schoberl (1947) and Schoberl and Wagner(1960).

APPENDIX 2

Thin Layer Chromatography and Electrophoresis

i Extraction of Tissue

100mg fresh weight of tissue was placed in a pre-chilled Potter-Elvehjem type glass homogeniser containing 2ml methanol/chloroform/water (12 + 5 + 3 v/v) (MCW) and the tube kept at -20°C for 30 minutes. The tissue was homogenised and the homogenate centrifuged for 10 minutes at high speed in an MSE Minor centrifuge at 4°C. The supernatent was transferred to a glass centrifuge tube, the residue homogenised again in 2ml MCW and centrifuged, and the supernatent added to the first extract.

To the combined supernatents was added 1ml chloroform and 1.5ml water and the extract mixed using a vortex mixer. The resulting two phases were separated by centrifugation for 15 minutes at high speed and the upper aqueous layer transferred to a 50ml volume pear-shaped flask. The extract was dried in a vacuum oven at a temperature not exceeding 30°C.

Dried extracts were stored successfully at -20°C for several months. The extract was dissolved in 10% iso-propanol to give a concentration of 400mg fresh weight ml^{-1}. Amino acids in solution could be stored for several months at 4°C.

ii Preparation of Thin Layer Plates

4mm, 20 x 20cm glass plates were well cleaned with scouring powder and soap and water, then wiped over with absolute alcohol to remove all traces of grease.

15g cellulose powder (Machery and Nagel, MN300) and 2.5g silica gel H (Merck) were homogenised with 100ml distilled water in a fast electric blender for 30 seconds. The mixture was allowed to stand for 30 seconds, mixed again for 30 seconds then allowed to stand for 60 seconds before spreading onto plates at a thickness of 300µm using a BTL motorized TLC coater. The plates were tapped on the side of the bench to smooth the surface, then allowed to dry first at room temperature in a horizontal position then in a vertical position overnight at 50°C. This ensured that the layer adhered to the plate without flaking off.

iii Loading of Thin Layer Plates

Extract equivalent to 10-20mg fresh weight of tissue was layered onto the plate in a thin band 2.5cm long and 2.5cm from the edges of the plate (Fig.A2.1) Extract was applied in 5 µl aliquots using a micro-capillary pipette, and the layer dried between each application. One small spot of a saturated solution of a yellow, marker amino acid, ε- (2:4 - dinitro-phenyl) - L - lysine hydrochloride, was placed at the origin.

iv Electrophoresis

The plate was sprayed lightly with electrophoresis buffer, pH2, containing 15.3ml formic acid (98-100%) and 57ml acetic acid in 1l distilled water. Excess buffer was removed by gently blotting the surface of the plate with chromatography

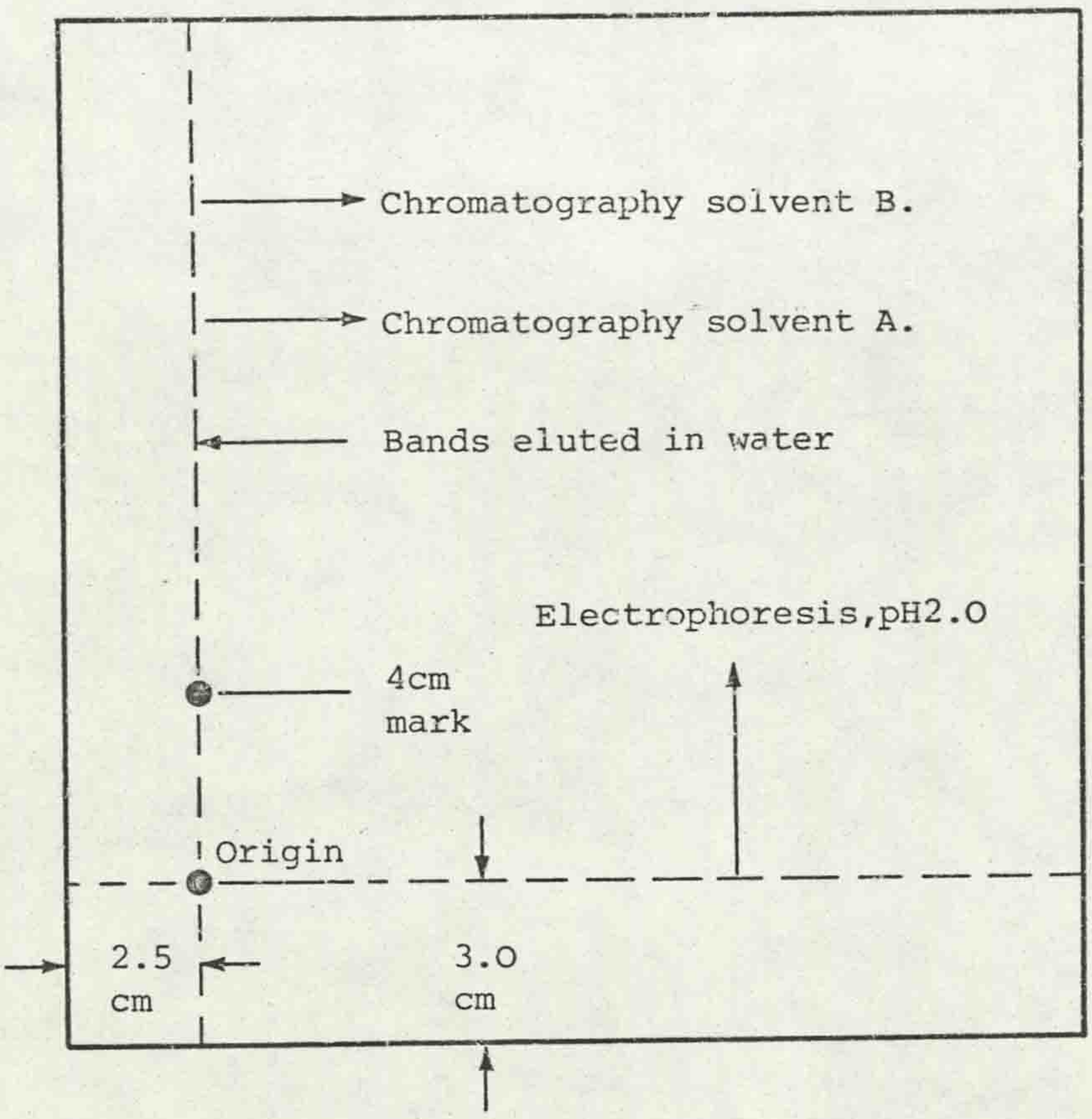


Fig.A2.1 Diagram of procedure used in two-dimensional separation of amino acids by high voltage electrophoresis and chromatography. (From Bieleski and Turner 1966)

paper, so the surface appeared translucent but not glistening. Plates were held horizontally to prevent streaming from the origin.

The plate was placed on the water-cooled base plate of the electrophoresis chamber (Shandon) with the origin at the anode. Wicks of buffer-soaked Whatman No. 3 chromatography paper were folded over onto the edges of the plate (Fig.A2.2) and secured with glass strips and a glass plate. This ensures good contact between the wicks and the thin layer.

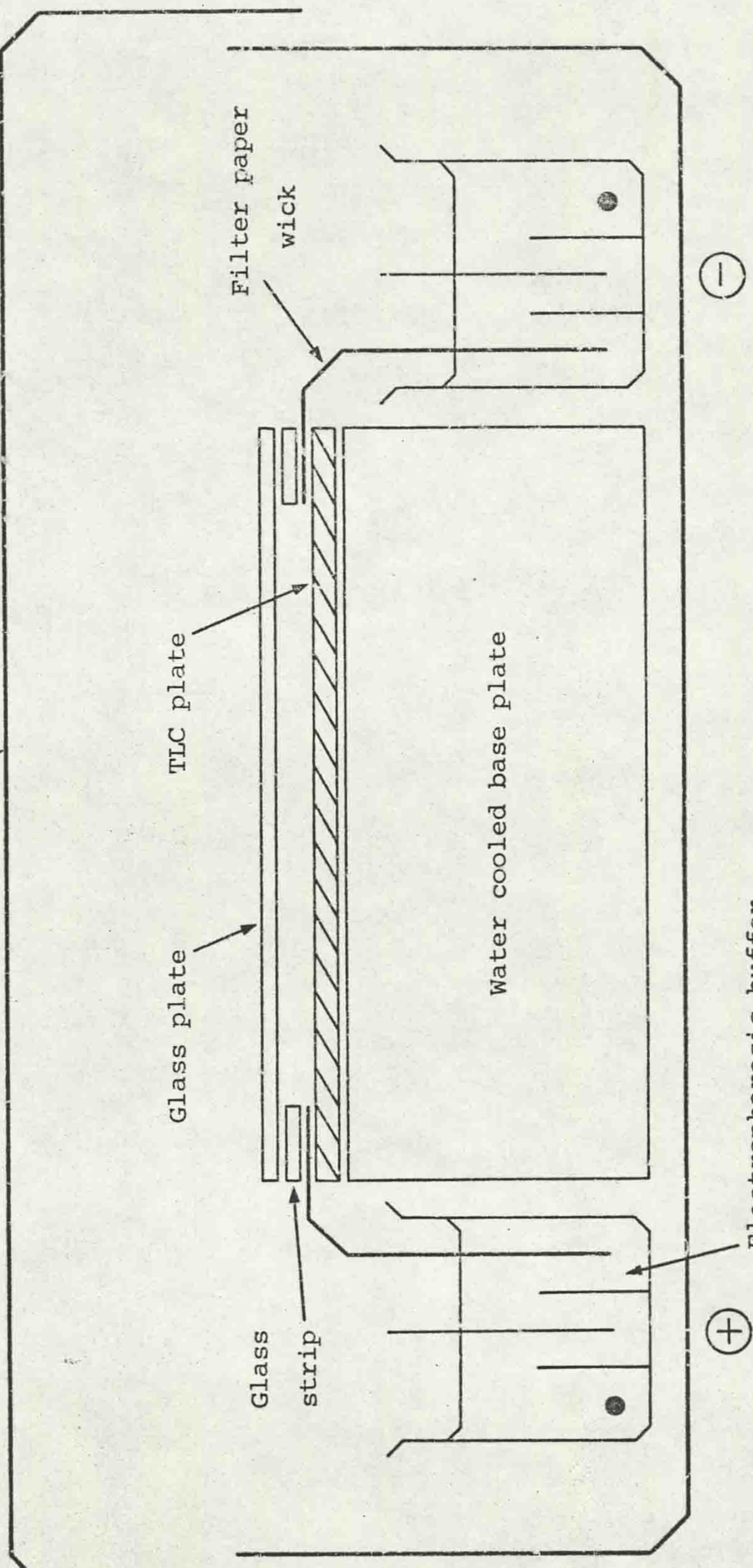
Electrophoresis was performed at 1000V and 20-40mA for approximately 25 minutes, until the yellow marker spot had reached the 4cm mark. This ensures optimum electrophoretic separation of the amino acids on a 20 x 20cm plate. After electrophoresis, the plate was dried in a stream of cool air blowing across the plate in parallel with the amino acid bands.

The amino acid bands were then reduced to spots by a water run, and the plate dried in a stream of cool air.

v Chromatography

The plate was then developed twice in the same direction, with methyl ethyl ketone/pyridine/water/acetic acid (70 + 15 + 15 + 2, v/v) and then n-propanol/water/ n-propyl acetate/acetic acid/pyridine (120 + 60 + 20 + 4 + 1, v/v). All solvents used for chromatography were of analytical grade (Analar and Aristar) to prevent contamination of the plates by trace impurities. Chromatography tanks were allowed to saturate for at least one hour before use.

Fig.A2.2 Schematic diagram of electrophoresis apparatus.



Plates were sprayed with 0.2% ninhydrin in acetone and the colour developed overnight at room temperature. All plates were run in duplicate and the pattern of amino acids obtained was recorded by tracing onto paper and by photography.

Standards of amino acids and their derivatives were run in various combinations and the results combined to produce a map of their distribution on thin layers under the conditions described.

The methods described here are modifications of those described by Bieleski and Turner (1966), Stahl (1969) and Granroth (1970).

APPENDIX 3

Plate A3.1 Autoradiograph of thin layer chromatogram of extracts of tissue supplied with ^{14}C-cysteine, a) onion callus (3 days), b) onion shoot tip (4 days).

a

b

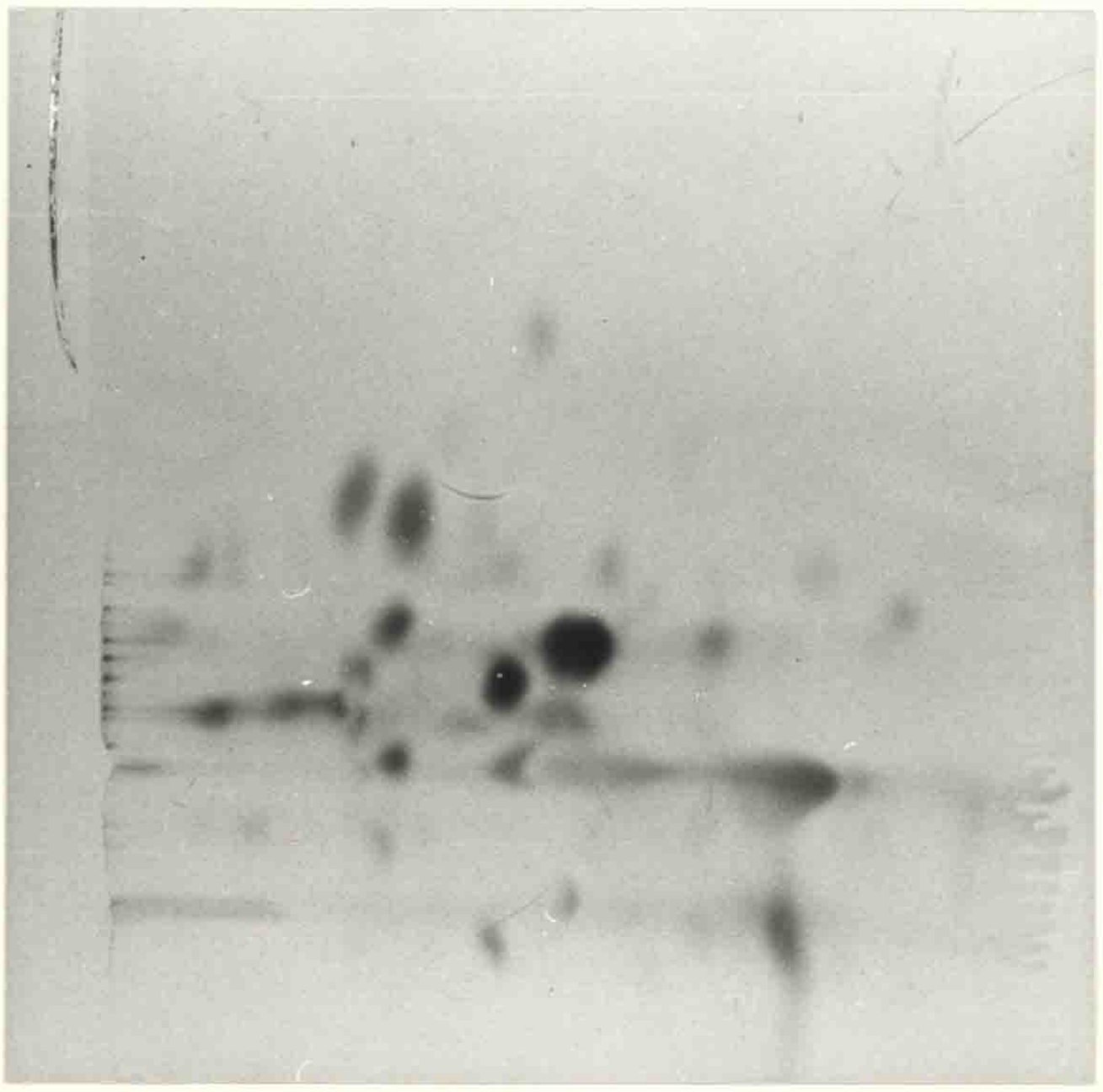


Plate A3.2 Autoradiograph of thin layer chromatogram of extracts of tissue supplied with ^{14}C-serine, a) onion callus (3 days), b) onion shoot tip (2 days).

a

b

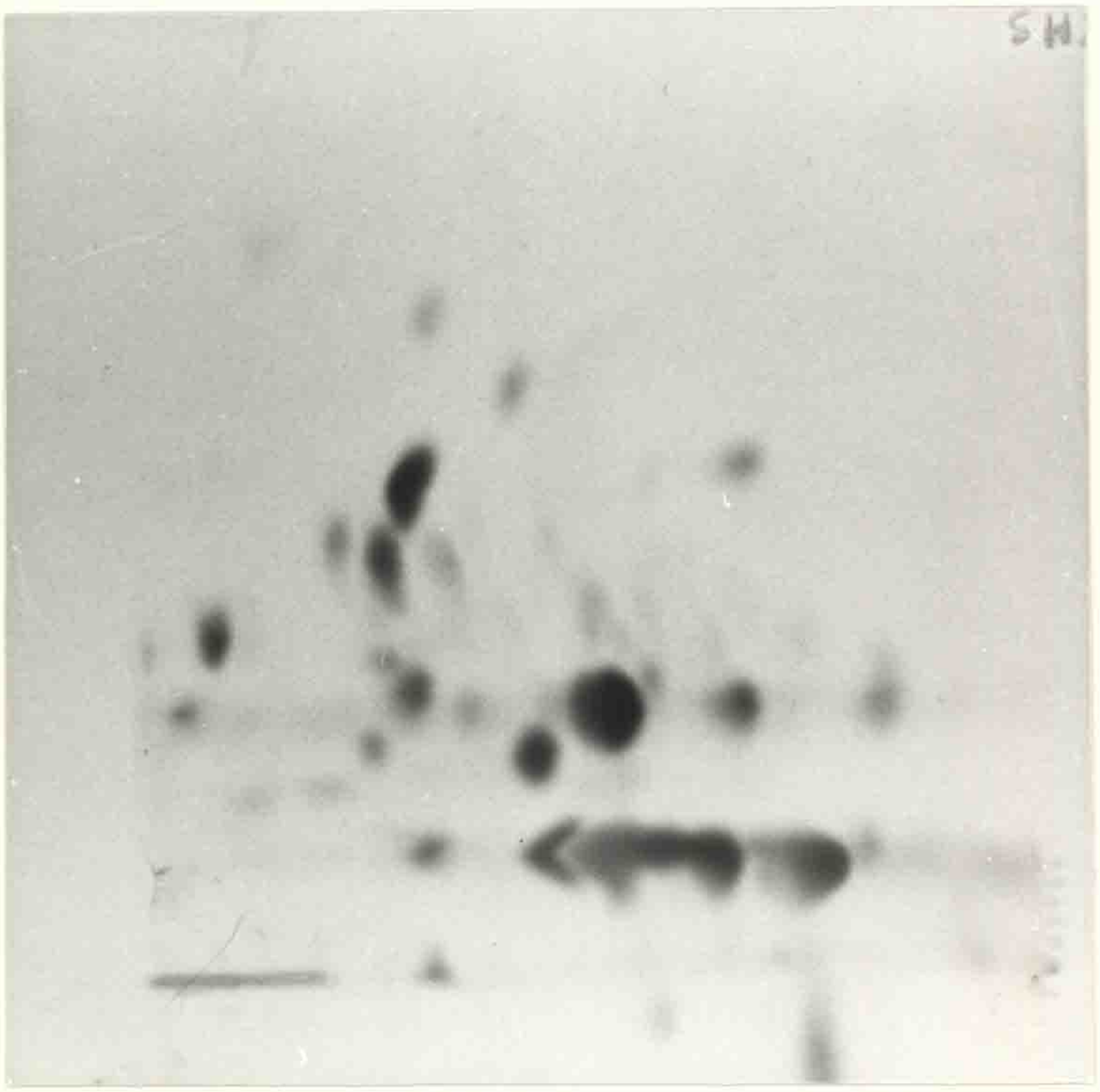
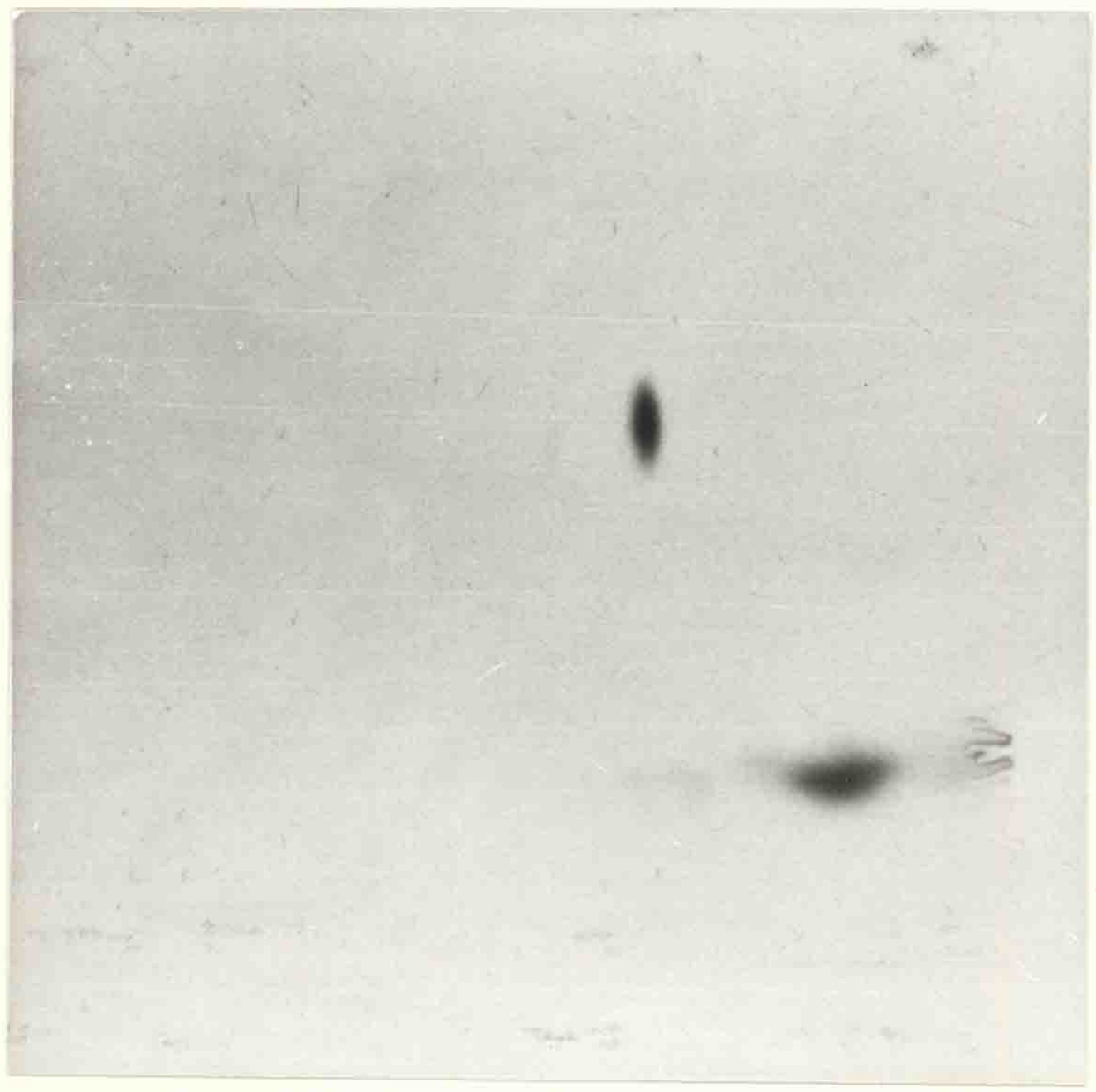


Plate A3.3 Autoradiograph of thin layer chromatogram of extracts of tissue supplied with ^{14}C-valine, a) onion callus (3 days), b) onion shoot tip (4 days).

a



b

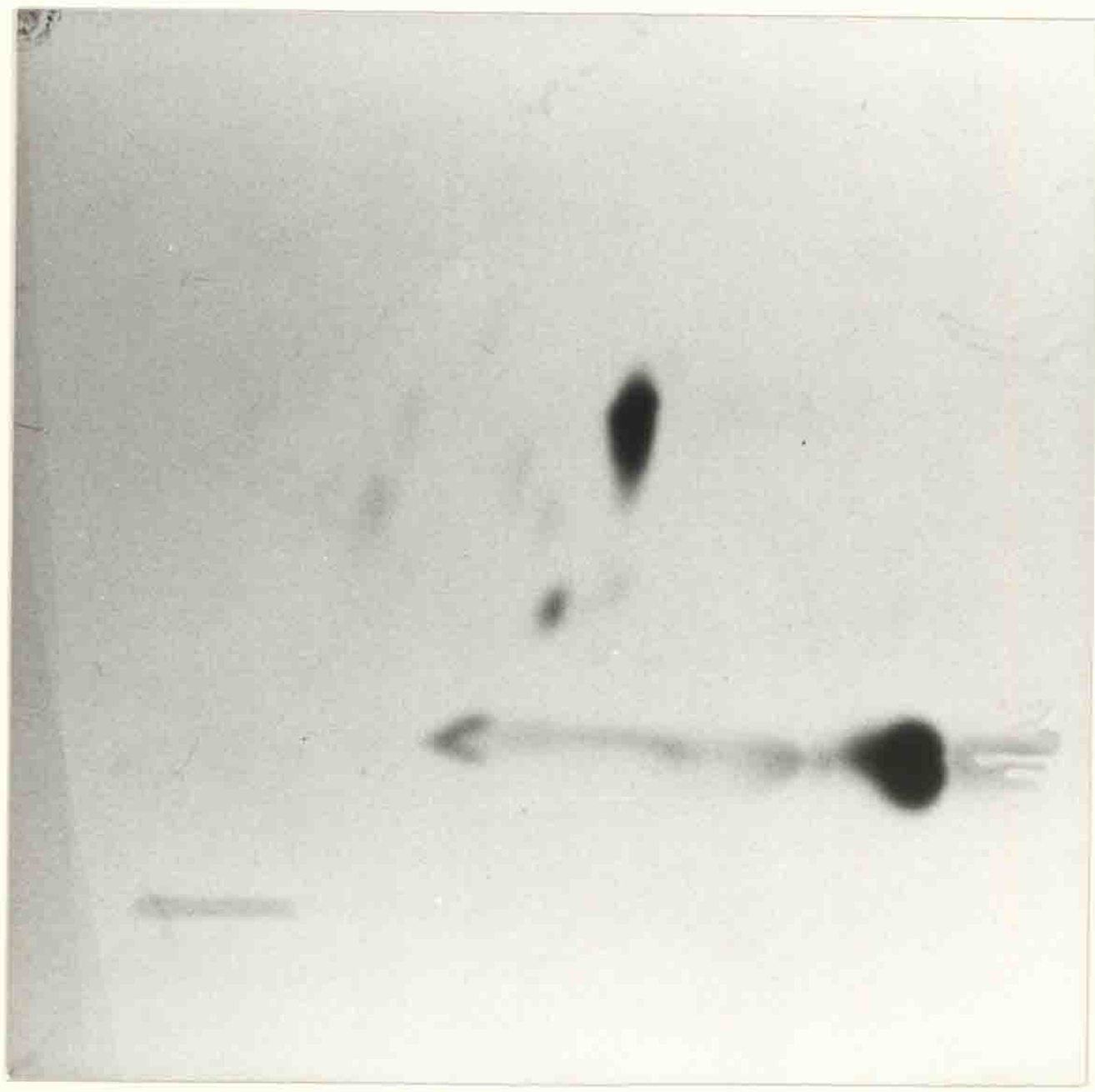
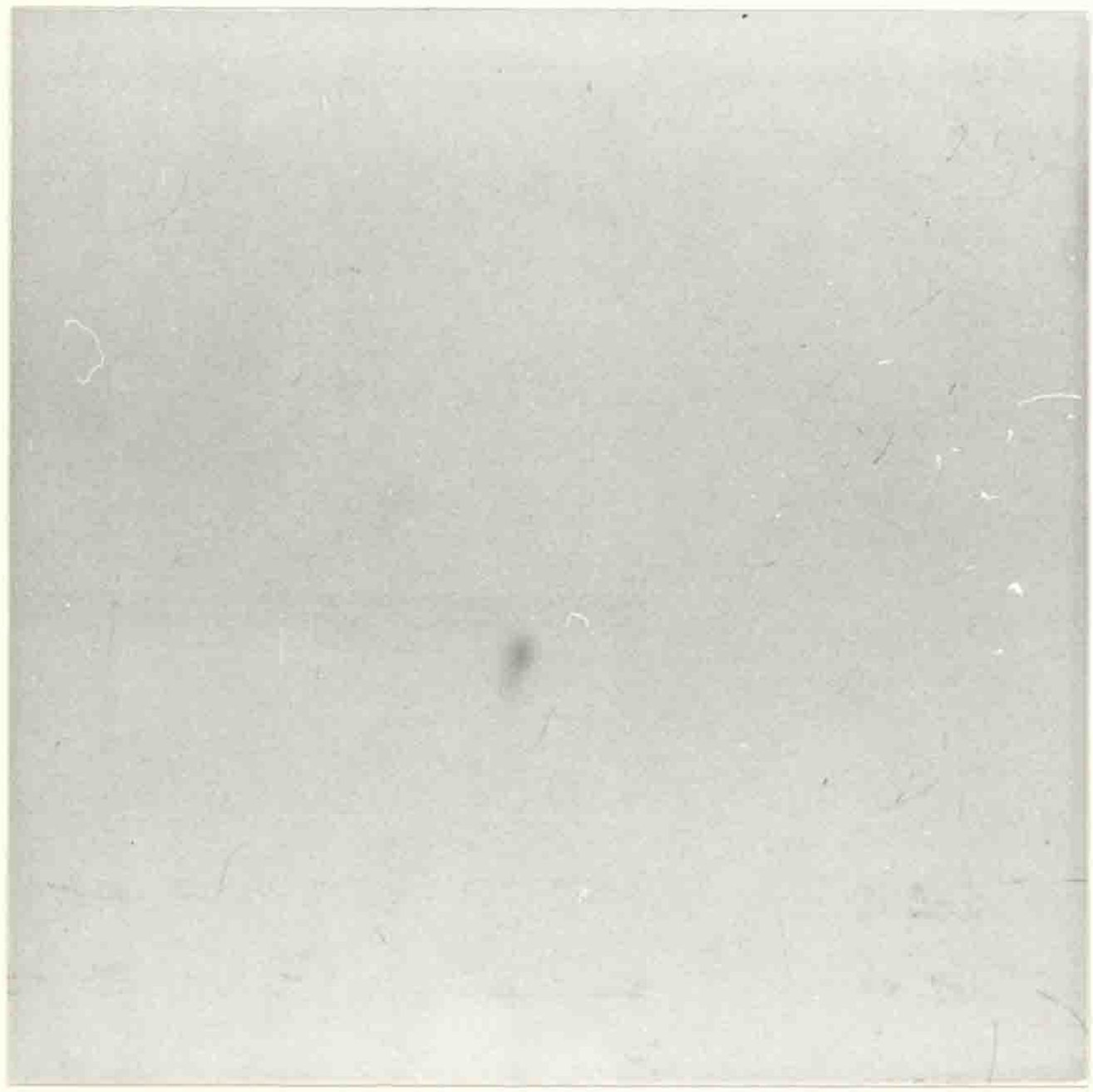


Plate A3.4 Autoradiograph of thin layer chromatogram of extracts from onion bulb slices fed with a) ^{14}C-cysteine, b) ^{14}C-valine

a



b

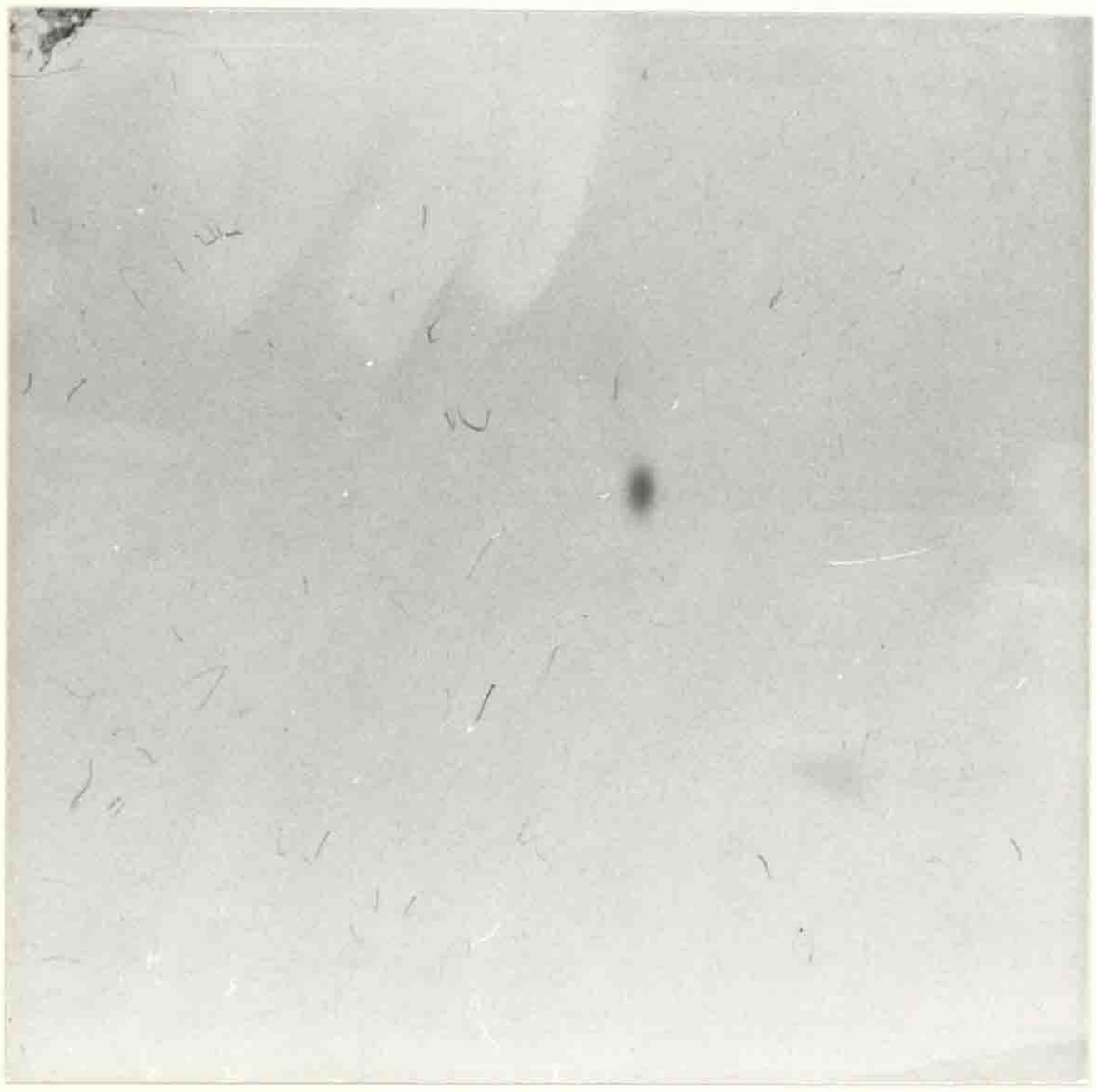


Table A3.1 Intensity of labelling of amino acids in callus, onion shoot tips and bulb slices after feeding ^{14}C - Cysteine.

Free amino acids and flavour compounds	Callus		Onion shoot tip		Onion bulb slice
	3 days	7 days	2 days	4 days	3 days
Lysine					
Arginine					
Glycine					
Alanine	*	*	*	*	
Valine		*	*	*	
Leucine			*		*
Isoleucine			*		*
Asparagine		*	*	**	
Threonine					
Serine			*	*	
Glutamine	*	*	*	**	*
Aspartic acid		*		**	
Glutamic acid	*	*	*	*	
Me Cy SO	**	**	**	**	
Pren Cy SO	**	**	**	**	*
Me Cys	*				
CPC	**	*	*	*	
Peptides	**	**	**	*	

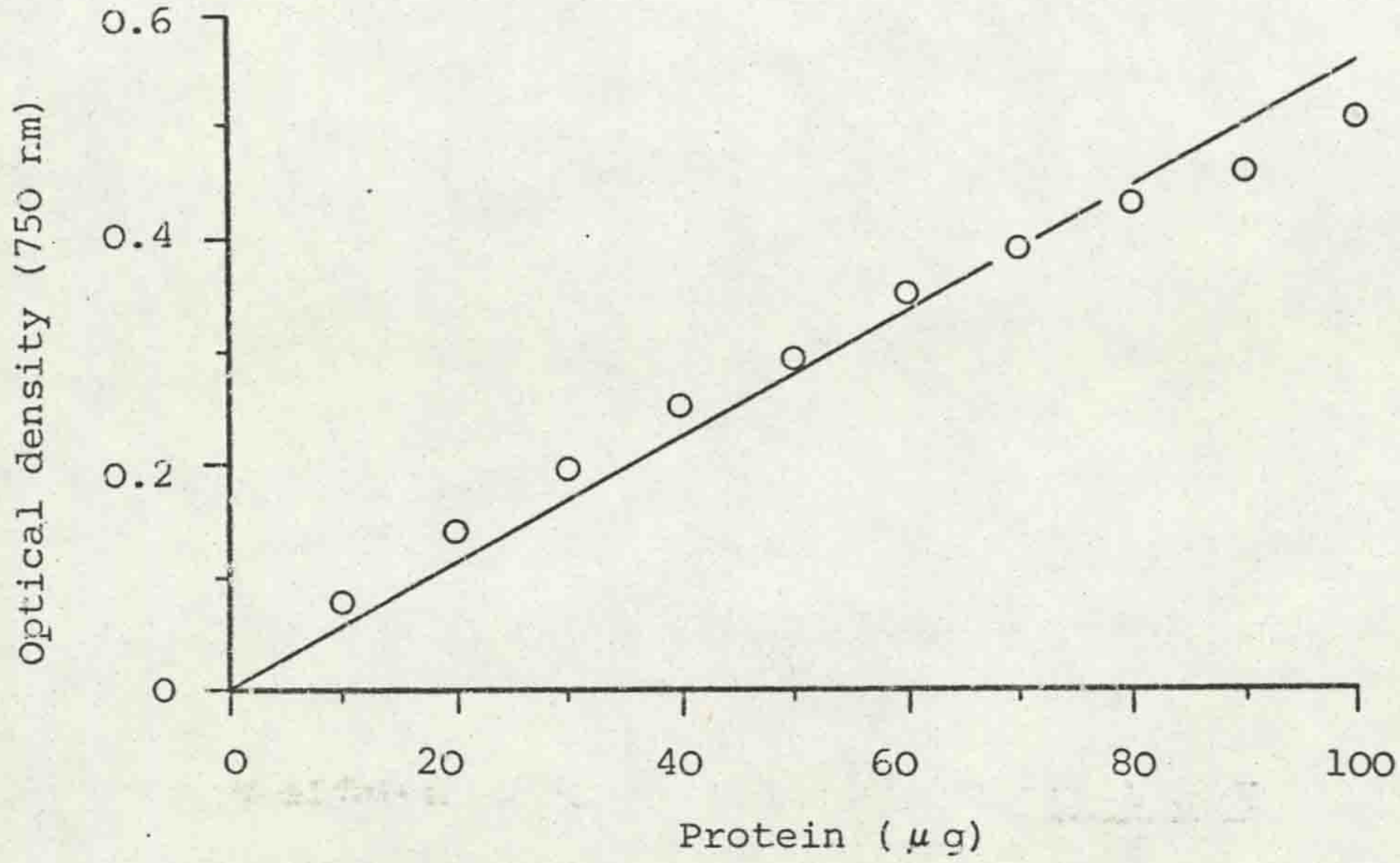
Table A3.2 Intensity of labelling of amino acids in callus and onion shoot tips after feeding ^{14}C - Serine.

Free amino acids and flavour compounds	Callus		Onion shoot tip	
	3 days	7 days	2days	4days
Lysine				
Arginine	*			*
Glycine	*	*	*	*
Alanine	*		*	*
Valine			*	*
Leucine	*		*	
Isoleucine	*		*	
Asparagine	*		*	*
Threonine			*	*
Serine	**	**	**	**
Glutamine	*	*	**	*
Aspartic acid			*	*
Glutamic acid	*	*	*	*
Me Cy SO	**	**	**	**
Pren Cy SO	*	*	**	**
Me Cys	*	*		*
CPC		*	*	*
Peptides	**	**	**	**

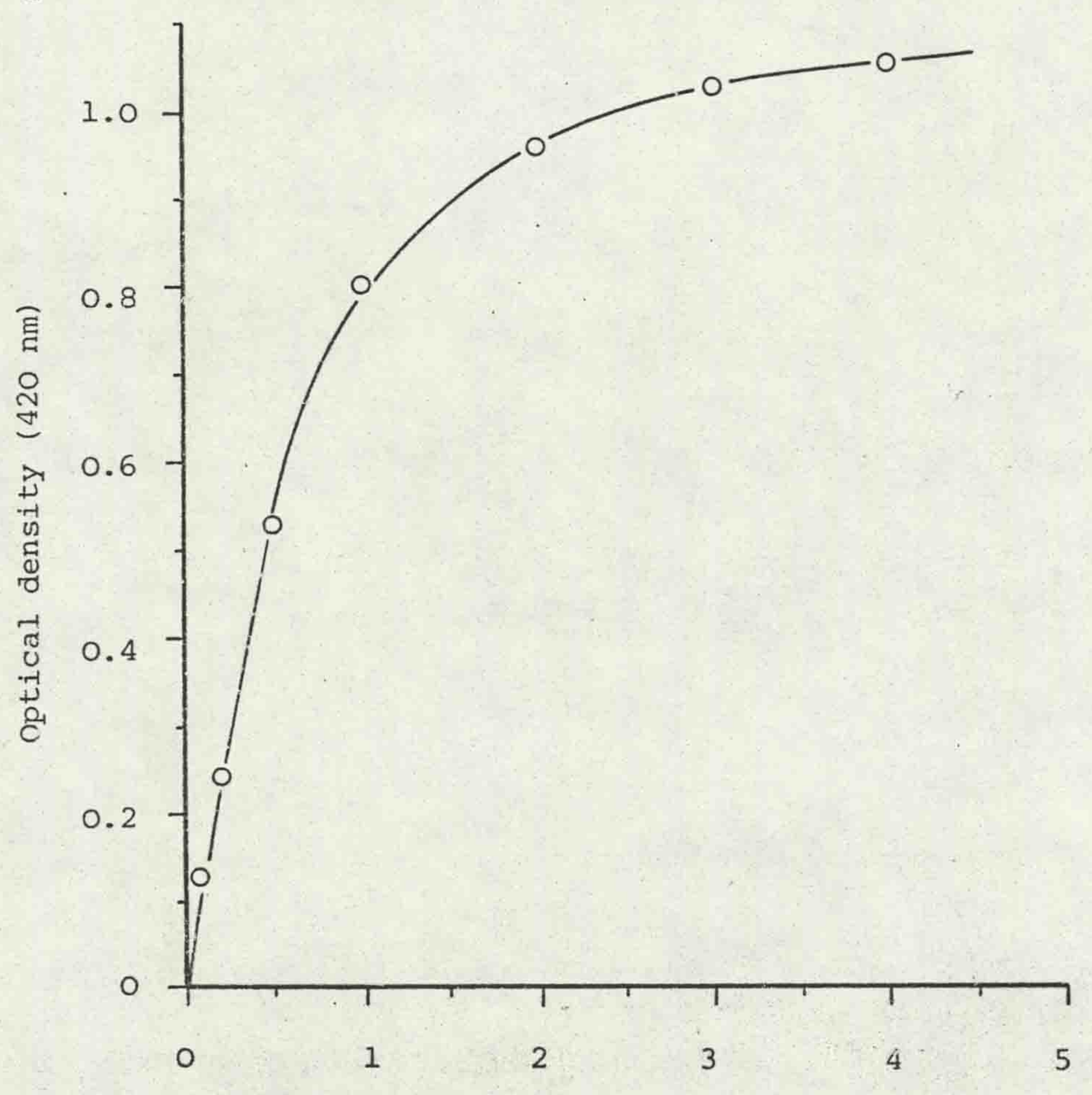
APPENDIX 4

Fig. A4.1 Standard curves for a) soluble protein using the Folin method of Lowry et al. (1951), b) pyruvate using the method of Schwimmer and Guadagni (1962).

a


Optical density (750 nm)
0.6
0.4
0.2
0
0
20
40
60
80
100
Protein (μ g)

b


Optical density (420 nm)
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Pyruvate (μ moles)

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List of Publications.

Selby, C., Turnbull, A. and Collin, H.A. Comparison of the onion plant (*Allium cepa*) and onion tissue cultures. II. Stimulation of flavour precursor synthesis in onion tissue cultures. (Submitted).

Turnbull, A., Smith, J.L. and Collin, H.A. Comparison of the onion plant (*Allium cepa*) and onion tissue cultures. III. Fine structure of onion bulb and callus cells. (Submitted).

Turnbull, A., Galpin, I.J. and Collin, H.A. Comparison of the onion plant (*Allium cepa*) and onion tissue cultures. IV. Biosynthesis of flavour precursors.(In preparation).

Turnbull, A. and Collin, H.A. Comparison of the onion plant (*Allium cepa*) and onion tissue cultures. V. Flavour production during root and shoot formation. (In preparation).

Turnbull, A. and Collin, H.A. 'Flavour production in onion tissue cultures.' Poster demonstration and abstract at the 12th Meeting of the Federation of European Biochemical Societies, Dresden, D.D.R. July 1978.

Turnbull, A. and Collin, H.A. 'Flavour production in onion tissue cultures.' Poster demonstration and abstract at the 4th International Congress of Plant Tissue and Cell Culture, Calgary, Canada. August 1978.